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# OXIDATIVE STRESS AND PROTEIN OXIDATION



Oxygen is a fundamental component of aerobic life. Molecular oxygen offers the opportunity for respiration, which is energetically more efficient than fermentation. However, the switch to an oxidative atmosphere was a source of massive environmental stress on existing life, forcing all organisms to adapt (1).

Biological systems are frequently exposed to reactive oxygen species (ROS) and reactive nitrogen species (RNS) which are generated exogenously as pollutants in the atmosphere (photochemical smog, ozone, pesticides, xenobiotics), during exposure to ultraviolet (UV) irradiation, X- or γ-rays, and endogenously as by-products of mitochondria-catalyzed electron transport reactions; products of oxidase-catalyzed reactions such as cytochrome P450 (CYP450) detoxification reactions; generated by metal-catalyzed reactions; products of arginine metabolism; and produced by neutrophils and macrophages during inflammatory conditions such as phagocytic oxidative bursts and peroxisomal leakage (2–4). In addition, these species play a role in a series of pathological situations, including atherosclerosis, rheumatoid arthritis, and other chronic inflammatory diseases; cancer, cataract, diabetes, and diabetic retinopathy; or neurodegenerative disorders such as Parkinson's disease (PD) and Alzheimer's disease (AD), as well as aging (5, 6).

Oxidative stress causes damage reactions which are mediated by a small fraction of the total oxygen consumed. This small percentage of the consumed oxygen is transformed to activated oxygen by-products, which might cause oxidative damage to biological molecules. However, a system of antioxidant

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defenses acts protectively to oppose the oxidative damage and is aimed to quench some reactive intermediates. Oxidative damage and antioxidant defense are normally in a more or less dynamic equilibrium. Often in the oxidative damage to biological molecules, trace elements such as iron or copper are involved (7).

The defense against ROS-mediated oxidative damage in all organisms is catalyzed by a large variety of different antioxidant defense systems which can either prevent the formation of these ROS/RNS or convert the most reactive metabolites into less active ones or inactivate these derivatives totally. These systems include a number of enzymes as major components, such as the superoxide dismutases (SODs), catalases (CAT), glutathione peroxidases (GPxs), reductases, and glutathione-S-transferases (GST); and a number of other thiol-specific enzymes, methionine sulfoxide reductases (MSR), and thioredoxin (Trx) reductases. Several metal-binding proteins such as ceruloplasmin, ferritin, and transferrin are also considered to be involved in the antioxidative defense, along with a number metabolites and cofactors ($NADP^+$/nicotinamide adenine dinucleotide phosphate [NADPH], $NAD^+$/nicotinamide adenine dinucleotide [NADH], lipoic acid, uric acid [UA], bilirubin, etc.), and some dietary components (vitamins C and E), and some trace metals as $Mg^{2+}$, $Mn^{2+}$, or $Zn^{2+}$ (8). Interestingly, it seems to be established that the ability to cope with ROS decreases with age in most cells, tissues, or organisms. Moreover, it seems to be established that a continuous low-level exposure of some, if not all, of these ROS may be involved in the regulation, induction, and maintenance of a number of biological functions (9) by regulating diverse cell signaling events (10, 11). However, overwhelmingly high concentrations of ROS can oxidize nucleic acids, lipids, and proteins. Much of the damage can be repaired; however, if unrepaired, oxidized DNA and RNA can lead to transcription/translation errors, and consequently lead to the synthesis of abnormal proteins, which in turn might not be only nonfunctional, but can also be more sensitive to oxidation by ROS (12, 13). The ability to counteract oxidative stress situations is declining with age, thereby causing higher vulnerability of older cells, tissues, and organisms to oxidative damage (14). ROS are potentially able to attack all cellular structures; however, the reactivity of the substrates might differ in dependence of the ROS and the target molecule generated, but in principle, macromolecules such as lipids, DNA, and proteins are major targets (15).

The interaction of ROS with lipids is generally known as a process called lipid peroxidation (LPO). This process might lead to the loss of membrane integrity and hence compromise several cellular functions, including signaling events such as the activation of the phospholipases $A_2$ mediated by changes in the membrane structure and composition. Activation of the phospholipase, in turn, leads to an increase in an influx of $Ca^{2+}$ ions and activation of further downstream molecules, including lipoxygenases (LOXs). Interestingly, LOX transforms polyunsaturated fatty acids (PUFAs) into lipid hydroperoxides (LOOHs). These reactions are generally catalyzed by these enzymes in a

highly selective and specific manner. Additionally, LOOHs are also formed by nonenzymatic LPO processes. Furthermore, intermediate products of LPO are formed by the decomposition of the hydroperoxides, resulting in some LPO products such as epoxides and highly unsaturated aldehydic compounds, which are of high chemical reactivity and difficult to detect (16).

LPO seems to be involved in a gradual cell damage occurring in some chronic diseases, for example in diabetes, rheumatism, atherosclerosis, and in the aging process itself. Importantly, dying cells in injured tissue also release enzymes able to facilitate the nonenzymatic LPO process. Some scenarios include the cleavage of membranes by esterases, the release of free unsaturated fatty acids, which are prone to enzymatic and nonenzymatic LPO, and the formation of LOOHs. In a series of nonenzymatic steps involving iron ions, these LOOHs decompose (17). For example, Das et al. demonstrated high LPO levels in the tissues of different-aged pigs with high concentrations of PUFAs in phospholipids of the membranes (18).

Besides LPO, one of the most significant consequences of oxidative stress is proposed to be oxidative DNA damage. However, due to the efficient DNA repair mechanisms, only a minor part of this DNA damage becomes permanent, forming mutations and/or genetic instability. Many different DNA base changes have been seen following some form of oxidative stress, and these lesions are widely considered as a first step in the development of cancer and are also implicated in the process of aging. The DNA repair mechanisms involved in the removal of oxidative DNA lesions are complex. For example, in Cockayne syndrome, characterized by premature aging, there appears to be deficiencies in the repair of oxidative DNA damage in the nuclear DNA, and this may be the major underlying cause of the disease (19). Oxidative damage to DNA causes not only strand breaks, but also the formation of specific base adducts, such as 8-hydroxy-2′-deoxyguanosine (7).

Accumulation of DNA lesions with age may be the underlying cause for age-associated diseases including cancer. ROS cause many types of DNA damage, including the abundant formation of 8-oxoguanine (8-oxoG) and thymine glycol (TG). 8-OxoG adopts a *syn* conformation and pairs with adenine, leading to transversion mutations, which may play a role in the development of cancer and the process of aging. In contrast, TG strongly blocks DNA replication and transcription and must be efficiently removed and repaired to maintain genetic stability. Base excision repair (BER) is the main excision repair system that removes 8-oxoG and TG. Persistent DNA damage can cause cell cycle arrest or induction of transcription, induction of signal transduction pathways, replication errors, and genomic instability (20).

Oxidative DNA damage accumulates with age in mitochondrial DNA rather than nuclear DNA. The mitochondrial theory of aging postulates that DNA damage and mutations accumulate in the mitochondrial genome, leading to mitochondrial dysfunction and cell death. Experimental data from several laboratories suggest that the amount of DNA damage such as 8-oxoG increases in the mitochondrial genome with age, and it is reported that mtDNA

from 23-month-old rat liver mitochondria has four times higher 8-oxoG than mtDNA from 6-month-old animals. In contrast, no significant change in the level of 8-oxoG was found in nuclear DNA from the same animals. In the same study, it has also been proposed that DNA repair capacity declines with age (21).

DNA repair is obviously a crucial function necessary to maintain genomic stability and function, so there is a need to understand whether and how mtDNA undergoes repair. Interestingly, the DNA repair activity appears to increase with age in the mitochondria, whereas it declines in the nucleus. Even with this increased DNA repair, there is still an increase in oxidative DNA base lesions observed in the mitochondria with age. This suggests that the oxidative DNA damage in mitochondria is dramatically increased during aging, which cannot be overcome by repair enzymes, although with higher activities.

BER is the major mechanism for the correction of damaged nucleobases resulting from alkylation and oxidation of DNA (22). Mitochondria have an efficient BER repair capacity, but cannot repair most bulky lesions normally repaired by nucleotide excision repair (NER) (21). The first step in the BER pathway consists of excision of the abnormal base by several specific DNA-N-glycosylases. A decrease in BER activity was found to be related to an increased risk of carcinogenesis and aging. To investigate BER activities in more detail, a new technique was developed to analyze DNA repair based on surface plasmon resonance imaging (SPRi) (22).

Base damage is often caused by ROS, for example, hydroxyl radical ($HO^{\bullet}$), superoxide radical ($O_2^{\bullet}$), and hydrogen peroxide ($H_2O_2$). Besides the repair mechanisms previously mentioned, another line of defense is the repair of oxidative damage in DNA by the intricate network of DNA repair mechanisms. Transcription-coupled repair (TCR), global genome repair (GGR), mismatch repair (MMR), translesion synthesis (TLS), homologous recombination (HR), and nonhomologous end-joining (NHEJ) also contribute somehow to the repair of oxidative DNA damage. TCR and MMR are also important backup pathways for the repair of transcribed strands and newly replicated strands, respectively (23).

Besides lipids and DNA, proteins are also prevalent targets for ROS-mediated oxidative damage. Many years ago, proteins have been recognized as major targets of oxidative modification, and the accumulation of oxidized proteins is a characteristic feature of aging cells. Moreover, in particular, proteins show age-dependent changes in their steady-state levels, considered as a part of developmental biology (24). The age-related accumulation of oxidized proteins is dependent on the balance between the generation of oxidatively modified proteins and their elimination by protein degradation and repair systems. During the last years of research, an increase in the amount of oxidized proteins has been described in many experimental aging models, often measured by the accumulation of protein carbonyls, tyrosine oxidation products, or by the accumulation of protein-containing pigments such as lipofuscin (15).

Many factors influence the level of protein damage induced by ROS. It is worth noting that this includes, of course, the nature and concentration of the ROS, the availability of the target protein, and the presence and functionality of antioxidant enzymes and compounds (25).

Oxidative protein damage plays a crucial role in cellular functionality since the oxidized proteins lose their catalytic functions. Therefore, the oxidative damage to a specific protein, might lead to more or less a pronounced loss of a particular biochemical function. It is required to mention here that such an oxidative protein damage might be mediated directly by ROS/RNS, but also by the secondary reactions via other by-products of oxidative stress. Examples of such secondary modification include carbohydrates and lipids modified by oxidative stress (including LPO products). Often studied examples of such reactive intermediate metabolites are the LPO products malondialdehyde (MDA) or 4-hydroxynonenal (HNE). A number of studies demonstrated the cross-linking and inactivation effects of these molecules on proteins (26). Besides the "classical" oxidation of proteins by ROS/RNS, the reaction with reducing sugars is also modifying proteins readily. This nonenzymatic glycation/glycoxidation process is also called the Maillard reaction or nonenzymatic glycosylation. In this nonenzymatic reaction, reducing sugars interact with proteins, often first by a reaction leading to the formation of Schiff base, that is, an imine double bond between the aldehyde group of the sugar and the amino group in the protein, often the epsilon amino group of lysine residues. Such an imine is quickly rearranged to form a ketoamine, forming a so-called Amadori product. Further reactions of the Amadori products lead to the irreversible formation of advanced glycation end products (AGEs) (2). Many of the reactions are site specific and influenced by reduction–oxidation (redox) cycling metals, mainly iron or copper. Generally, the classification of the oxidative modifications is done by separating the reactions into those which oxidize and cleave the peptide bond and those which modify side chains. Oxidative protein modifications are also divided into specific and global ones. In the specific modifications such as dityrosine formation, less residues or proteins are affected, while in the global ones such as carbonyl formation, a more or less substantial fraction of proteins might be affected in the sample (24).

Several types of ROS-induced protein modifications have been demonstrated, including the loss of sulfhydryl (-SH) groups, formation of carbonyls, disulfide cross-links, dityrosine cross-links, nitrotyrosine, and glyoxidation and LPO adducts, among others (27). Some modifications listed here are: the oxidation of leucine resulting in the formation of various hydroxyleucines; tryptophan oxidation to form N-formylkynurenine, kynurenine, and further downstream products; histidine oxidation to form aspartate or asparagine; phenylalanine oxidation to ortho- and metatyrosine; tyrosine oxidation to form 3,4-dihydroxyphenylalanine (DOPA), dityrosine, 3-chlorotyrosine, and 3-nitrotyrosine (3-NY); and methionine (Met) oxidation to form methionine sulfoxide (MetSO). Some of the most commonly measured protein oxidation products of Val, Leu, Ile, Lys, Glu, Arg, and Pro are alcohols and carbonyl

groups (28). Protein carbonyl groups have the advantage of being abundantly formed on a (theoretically) low background of carbonyl groups in nonoxidized proteins, resulting from the few carbonyl-bearing enzymatically introduced posttranslational modifications. However, protein carbonyls have the disadvantage of being nonspecific oxidation markers. In addition to the modification of amino acid side chains, oxidation reactions can also lead to a fragmentation of polypeptide chains, or to the formation of protein aggregates by intermolecular cross-linking of peptides and proteins (29). Other nonenzymatic processes can also contribute to protein modification: as $N^{\varepsilon}$-carboxymethyllysine ($N^{\varepsilon}$-CML), pentosidine, and a range of compounds called AGEs, as already mentioned (28).

It was long assumed that oxidation of many proteins is a random process, however certain proteins or protein domains seem to be oxidized preferentially or accumulate in the oxidized form more easily. Recently, the existence of site-specific oxidation processes is discussed more and more. This is supported by selective findings that some enzymes have been observed due to accumulating in an oxidized form during aging (30, 31), for example, glutamine synthetase (GS) (32, 33), mitochondrial aconitase (34), adenine nucleotide translocase (35) and calcineurin (36), glucose-6-phosphate dehydrogenase (G6PDH) (14), tyrosine hydroxylase (37), and some enzymes of the antioxidant defense system (38, 39). Interestingly, the elongation factor 2 (EF-2) was found to be oxidized during aging, a process that is proposed to lead partially to the decline of protein synthesis during aging (40).

Accumulation of oxidized proteins is a complex process dependent on the formation rates of different ROS species, the levels of numerous antioxidative systems, and the rates of degradation of oxidized proteins by a multiplicity of proteases that have been shown to decline during aging. Because the cellular levels of oxidized proteins are dependent upon so many variables, the mechanisms responsible for the accumulation of oxidatively modified proteins in one individual may be very different from those involved in another individual (29). Interestingly, certain oxidation processes of cysteine (Cys) and Met are reversible due to the existence of specific enzymatic systems, which can bring these modifications back to the reduced form. Irreversible oxidation products of other amino acids are most frequently hydroxylated and carbonylated amino acid derivatives. Oxidized proteins are generally less active, less thermostable, and have exposed hydrophobic amino acids at their surface (41). In order to be removed from the cellular protein pool, proteins harboring such irreversible amino acid modifications have to be degraded.

However, aging is accompanied by a loss of the cellular proteolytic activity and hence a further increase in the accumulation of damaged proteins and more thermolabile and catalytically inactive enzymes.

Therefore, whereas oxidative damage to nucleic acids is subject to an efficient repair by highly efficient mechanisms, the repair of damage to proteins appears to be limited to the reduction of oxidized derivatives of the sulfur-containing amino acid residues, as already mentioned. The reason for that is the plethora of possible oxidation products of the 20 amino acids, obviously

exceeding in their numbers the range of an efficient repair. Therefore, the repair of other than sulfur-containing amino acids after protein oxidation has not been demonstrated. Hence, these damaged proteins are target for degradation by various intracellular proteases, including cathepsins, calpains, and especially the 20S proteasome (42, 43).

However, certain oxidized proteins are poorly handled by cells, and together with possible alterations in the rate of production of oxidized proteins, this may contribute to the observed accumulation and damaging actions of oxidized proteins during aging and in pathologies such as diabetes, atherosclerosis, and neurodegenerative diseases. Protein oxidation may also sometimes play controlling roles in cellular remodeling and cell growth. Proteins are also key targets in defensive cytolysis and in inflammatory self-damage (44).

## 1.1 THE LARGE VARIETY OF PROTEIN OXIDATION PRODUCTS

### 1.1.1 Primary Protein Oxidation Products

As previously mentioned, free radical and oxidant flux, as by-products of metabolic and energy transfer processes, are an inevitable hallmark of oxidative life. It is suggested that the formed reactive species react with cellular components, including proteins. Protein molecules containing such modified moieties may diffuse or be transported to other parts of the cell different from their origin, thus damaging more components due to secondary reactions (45).

Radicals react in a variety of reactions, including electron transfer (oxidation or reduction of the substrate), hydrogen abstraction, fragmentation and rearrangement, dimerization, disproportionation, and substitution (concerted addition and elimination) with amino acids, peptides, and proteins. The result of an interaction of a radical with a peptide is the formation of a peptide radical. The properties of the radicals formed on peptides and proteins depend on the nature and reactivity of the attacking radical. Thus, electrophilic radicals (e.g., $HO^{\bullet}$, alkoxyl radicals) preferentially oxidize electron-rich sites, whereas nucleophilic species (such as phenyl and many other carbon-centered radicals) attack electron-deficient sites (46). While the positional selectivity and rates of radical attack on free amino acids are relatively well characterized, the situation with peptides and proteins is less clear. There is a wide variation in the magnitude of the rate constants for attack by species such as $HO^{\bullet}$ on free amino acids, and this can be readily accounted for a preferential attack at sites remote from the deactivating (powerfully electron-withdrawing) protonated amine group at the $\alpha$-carbon of free amino acids, and the presence of radical stabilizing groups on some side chains. Furthermore, the deactivating effect of the protonated amino group is exerted over long distances, so that the attack on hydrocarbon side chains (e.g., Val, Leu, Ile) skewed toward the most remote sites (47–49). Thus, the ratio of an attack at potential sites is different

from that expected on the basis of the greater stability of tertiary > secondary > primary carbon-centered radicals arising from the increased number of electron-releasing (stabilizing) alkyl groups.

The selectivity of an attack on side chains is also markedly affected by the presence of a functional group which can stabilize the resulting radicals. Thus, hydrogen atom abstraction occurs preferentially at positions adjacent to electron-stabilizing groups such as hydroxy groups (in Ser and Thr), carboxyl and amide functions (in Asp, Glu, Asn, Gln), and the guanidine residue in Arg (46). In contrast, the protonated amine function on the Lys side chain has a similar effect as the protonated amine group on the α-carbon. This results in hydrogen abstraction at sites remote from both groups, and hence products arising mainly from the C4 and C5 positions on Lys (50, 51). Addition reactions are usually faster than hydrogen atom abstraction reactions, as there is no bond breaking involved in the transition state. Hence, addition to the aromatic rings of Phe, Tyr, Trp, and His, and the sulfur atoms of Met and Cys predominates over abstraction from the methylene (-CH2-) groups. The adduct species formed with the aromatic rings are stabilized by delocalization on to neighboring double bonds. The only major exception occurs with Cys, where hydrogen abstraction from the thiol (-SH) group is particularly fast (52).

The conversion of the deactivating amine group on the α-carbon into an (electron delocalizing) amide function through the formation of a peptide bond increases both the extent and rate constant for attack of radicals such as $HO^{\bullet}$ at the α-carbon, thereby resulting in significant levels of backbone oxidation (49, 53). The range of rate constants for $HO^{\bullet}$ attack on amino acid derivatives (e.g., N-acetylated species) or simple two amino acid peptides (e.g., the Gly-X series) is much smaller than that observed with the free amino acids (46).

The α-carbon radical formed as a result of hydrogen atom abstraction from the backbone is particularly stable as a result of electron delocalization on both the neighboring amide group (on the N-terminal side) and the carbonyl function (on the C-terminal side) (54). This has important consequences for radical transfer reactions. Not all α-carbon radicals are of equal stability, however, and there is evidence for preferential formation at Gly residues in peptides (55). This has been postulated to arise because of steric interactions between the side chain and backbone groups, which prevents the α-carbon radical from achieving planarity (and hence effective electron delocalization) for those residues with bulky side chains (56). This results in the secondary α-carbon radical formed from Gly being more stable than the tertiary α-carbon radical formed from other amino acids in peptides.

Secondary and tertiary structures may play a significant role in blocking access of radicals present to backbone sites as a result of the outward protuberance of the side chains. This would suggest that side chain reactions may play a more important role in the chemistry of intact globular or sheet proteins than in the chemistry of disordered structures or small random coil peptides (57).

ROS-mediated oxidation of amino acid side chains leads to the formation of 2-oxohistidine from histidine (58), but also to the unstable amino acids asparagine and aspartic acid (59); tryptophan residues oxidation leads to kynurenine or N-formylkynurenine (60); tyrosine residues lead to dihydroxy derivatives (61); Met residues lead to MetSO or methionine sulfone derivatives (62); leucine and valine residues lead to hydroxy derivatives; and Cys residues lead to disulfide derivatives (63). Of particular significance is the fact that oxidation of many of the proteinogenic amino acids (lysine, arginine, and proline residues) lead to the formation of carbonyl derivatives (64). Other products of ROS attack on proteins include hydroperoxides and alcohols. Particularly reactive is the hydroxyl radical, which can introduce hydroxyl groups into phenylalanine and tyrosine residues, and cleave the ring structure of tryptophan. A peptide bond cleavage may also occur (59).

Amino acid composition results from mutation–selection balance caused by the antagonism between mutational biases and the selective pressure to maintain protein function and structural stability (1). Toxicity of oxidized proteins is related to oxidative cleavage of the polypeptide chain, modification of amino acid side chains, generation of protein–protein cross-linkage, and formation of derivatives sensitive to proteolytic degradation (29).

The amino acids most susceptible to oxidation (histidine, tryptophan, methionine, tyrosine, and Cys) (65) would be avoided in highly oxidizing environments. This effect should be more prominent on amino acids subject to irreversible oxidation (histidine, tryptophan, and tyrosine) than on amino acids capable of reversible oxidation (Met and Cys) (1). Because oxygen diffuses through the membranes to enter the cell, membrane proteins are expected to show signs of adaptation to high oxygen concentrations.

$HO^{\bullet}$ was shown to be the most effective oxidant, whereas other species are more selective (but less efficiently in inactivation), such as $(SCN)_2^{\bullet-}$, $Br_2^{\bullet-}$, $Cl_2^{\bullet-}$, and $I_2^{\bullet-}$. For example, $(SCN)_2^{\bullet-}$ was found to react with a key tryptophan residue in pepsin and so inactivates the enzyme, although damage could be reversed by the same radical (44). Inactivation by hydrated electrons has also been reported (66), but its significance, and that of the previously mentioned selective radicals, for biological systems may be limited. In studies on D-amino acid oxidase, it was found that removal of the coenzyme FAD enhanced radical damage and inactivation, illustrating that conformation and ligands can affect the extent of inactivation (44).

However, proteins may differ strongly in their susceptibility to oxidative damage. The redox-sensitive amino acids of bovine serum albumin (BSA), for example, were shown to be oxidized about twice as fast as those of GS (67), and intact proteins are less sensitive to oxidation than misfolded proteins (12).

***1.1.1.1 Carbon-Centered Radicals*** These radicals may be produced at either side chains or α-carbon sites, following the reaction of radicals with large peptides and proteins. Carbon-centered radicals are generally formed

via radical addition to an aromatic ring, hydrogen abstraction from C-H bonds (side chain or α-carbon), or secondary reactions of alkoxyl-, peroxyl- (68), or nitrogen-centered radicals (69). Carbon-centered radicals formed on proteins dimerize in the absence of $O_2$, or can form peroxyl radicals in its presence (70) (Fig. 1.1). Peroxyl radical formation predominates against dimerization in the presence of $O_2$, because for dimerization two radicals are necessary and this might be sterically hindered. Peroxyl radicals can also be generated, in the absence of $O_2$, from metal ion-catalyzed decomposition of hydroperoxides (71, 72). In contrast, in the absence of $O_2$, some of the substituted carbon-centered radicals undergo slow unimolecular elimination reactions. Thus, α-hydroxyalkyl radicals with L-amino groups (e.g., those formed from Ser and Thr) can release $NH_3$. This process may occur with some side chain-derived radicals, for example those formed at C5 of 5-hydroxylysine.

Peroxyl radicals undergo a number of reactions that result in the formation of carbonyl groups (aldehydes or ketones), alcohols, and hydroperoxides. Peroxyl radicals undergo ready dimerization reactions with other peroxyl radicals or related species such as $O_2^{\bullet-}$/$HOO^{\bullet}$; reactions with the latter species are more likely with proteins for steric reasons (46).

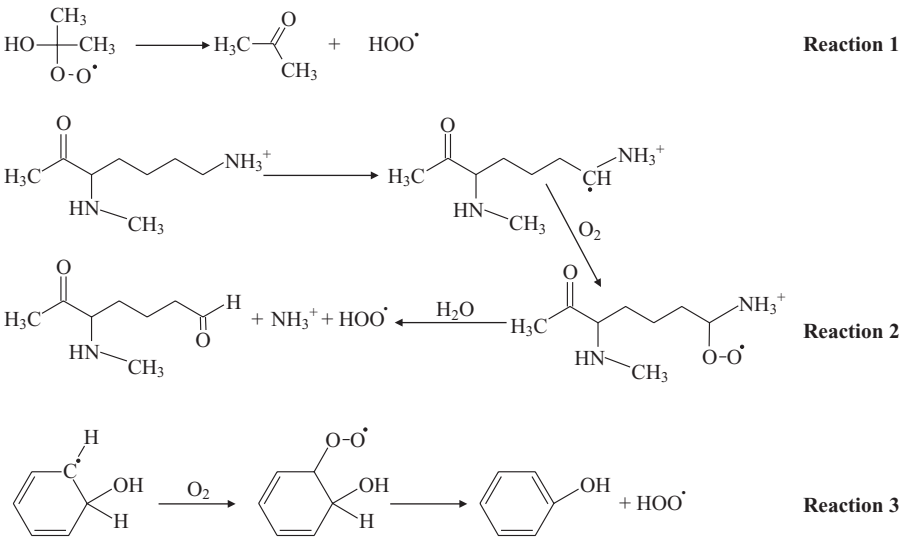



**FIG. 1.1** α-Substituted heteroatom containing peroxyl radicals may undergo a quick elimination of $HOO^{\bullet}$ or $O_2^{\bullet-}$ as shown in **reaction 1**. A carbon-centered radical as found in C6 of lysine side chains can react very fast with O2, releasing peroxyl radicals ($HOO^{\bullet}$) and $NH_3^+$ (**reaction 2**), forming an α-aminoadipate-δ-semialdehyde. Carbon-centered radicals of aromatic ring structure also form peroxyl radicals (**reaction 3**) (according to Clare et al., Biochim. Biophys. Acta 1504: 196–219, 2001).

α-Carbon peroxyl radicals undergo a complex series of reactions which result in backbone cleavage (63). These species have been assumed to rapidly eliminate $HOO^{\bullet}$ to give acyl imines that subsequently react with water to form the corresponding amides and carbonyl compounds. However, studies on cyclo($Gly_2$) and cyclo($Ala_2$) have shown that these peroxyl radicals undergo only a slow loss of $HOO^{\bullet}$. At high pH, ionization of the -NH- group (p$K_a$ 10.8 and 11.2 for cyclo($Gly_2$) and cyclo($Ala_2$), respectively) results in the rapid (base-catalyzed) elimination of $O_2^{\bullet-}$. This process gives a single product (Fig. 1.2, reaction 4), whereas at lower pH values, where slow loss of $HOO^{\bullet}$ is observed, bimolecular decay predominates and multiple species are formed (Fig. 1.2, reaction 5). Hydrogen atom abstraction by backbone α-carbon peroxyl radicals yields to α-carbon hydroperoxides, whereas cross-termination reactions with $O_2^{\bullet-}$ and $HOO^{\bullet}$ yields alkoxyl radicals (73).

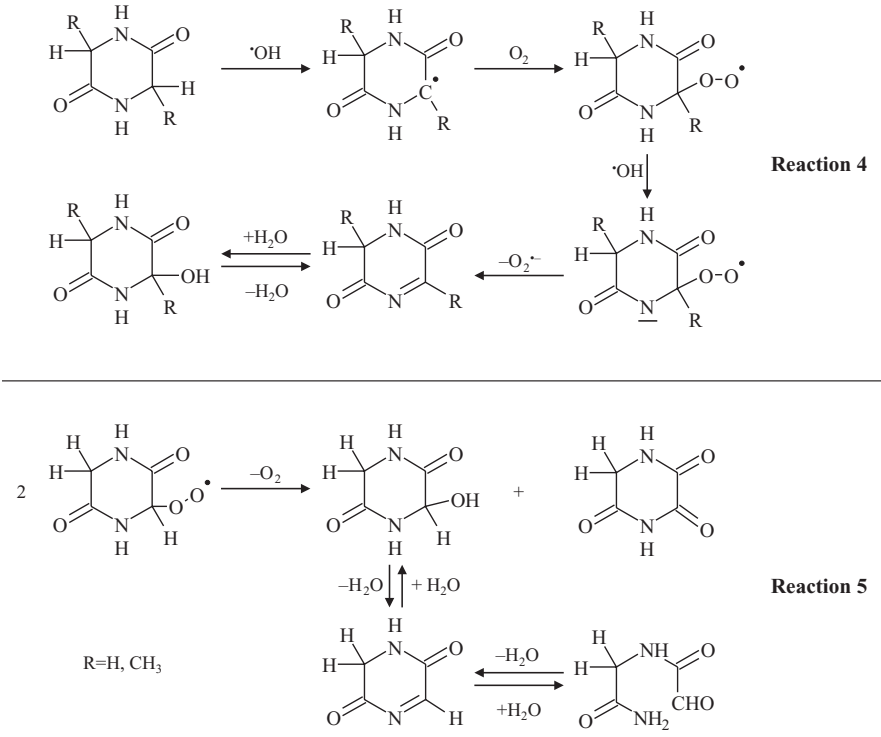



**FIG. 1.2** **Reaction 4** shows the reaction of cyclo($Gly_2$, R=H) or cyclo($Ala_2$, R=$CH_3$): after abstraction of an H-atom by an $^{\bullet}OH$ radical followed by reaction with $O_2$, the resulting peroxyl radical releases $O_2^{\bullet-}$ very quickly at high pH. At lower pH, release of hydroperoxyl ($^{\bullet}OOH$) happens at a much slower rate and different products are formed (**reaction 5**) (according to Clare et al., Biochim. Biophys. Acta 1504: 196–219, 2001).

Side-chain aminyl radicals (e.g., those formed from the ε-amino group of Lys side chains) undergo intramolecular abstraction, and this generates carbon-centered radicals at either C3 or the α-carbon (69).

Hydrogen abstraction from the α-carbon position accounts for more than 90% of the radicals formed with a series of alanine-derived peptides on reaction with $HO^{\bullet}$. This is due to the greater stability of the α-carbon radical over the primary alkyl radical formed on hydrogen atom abstraction from the methyl side chain (74). However, the yield of such backbone-derived radicals decreases markedly when there are side chains present, which can form stabilized radicals, or when steric factors play a role. α-Carbon radicals decay mainly by dimerization in the absence of $O_2$ (75). In the former case, significant yields of cross-links involving side chain-derived radicals have been identified; in the presence of $O_2$, peroxyl and alkoxyl species are also generated (76).

α-Carbon-centered radicals are also generated on addition of the solvated electron to backbone carbonyl groups (Fig. 1.3, reaction 6). The resulting midchain α-hydroxy α-amido radicals, formed on protonation of the initial adduct, decay primarily via reaction with other radicals in the absence of $O_2$. Thus, the reaction with an α-carbon radical results in the repair of both species (Fig. 1.3, reaction 7). The initial adduct species also undergo electron transfer reactions with acceptors such as disulfide (Fig. 1.3, reaction 8) or His residues. The main chain cleavage via reaction 9 (Fig. 1.3) is believed to be a minor process.

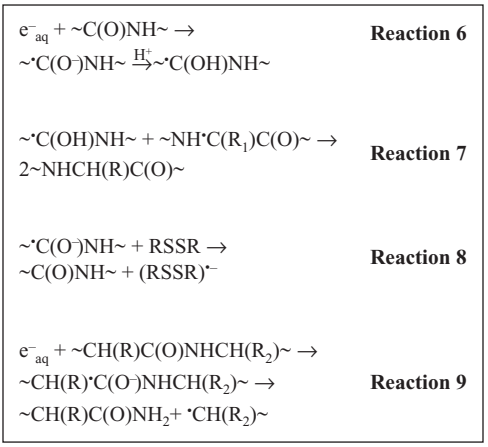



**FIG. 1.3** After addition of a solvated electron, an α-carbon-centered radical is generated at the backbone carbon (**reaction 6**). The formed midchain α-hydroxy α-amido radical decays in the absence of $O_2$ via the reaction with other radicals (**reaction 7**), in this case resulting in the repair of both species. Electron transfer reactions are possible between disulfides or histidine residues (**reaction 8**). A cleavage of the main chain is a minor process in this scenario (**reaction 9**) (according to Clare et al., Biochim. Biophys. Acta 1504: 196–219, 2001).

| | |
|---|---|
| $RS\text{-}SR + light \rightarrow 2\ RS^{\bullet}$ | **Reaction 10** |
| $RS\text{-}SR + e^{-} \rightarrow RS^{\bullet} + RS^{-}$ | **Reaction 11** |

**FIG. 1.4** Radical formation by photooxidation (**reaction 10**) or reduction (i.e., electron absorption) (**reaction 11**) of a disulfide bond (according to Clare et al., Biochim. Biophys. Acta 1504: 196–219, 2001).

| | |
|---|---|
| $RS^{\bullet} + O_2 \rightarrow RSOO^{\bullet}$ | **Reaction 12** |
| $RS^{\bullet} + RS^{-} \rightarrow (RSSR)^{\bullet -}$ | **Reaction 13** |
| $(RSSR)^{\bullet -} + O_2 \rightarrow RSSR + O_2^{\bullet -}$ | **Reaction 14** |

**FIG. 1.5** Formation of a peroxyl radical by $O_2$ absorption of a thiyl radical (**reaction 12**), reaction of a thiol anion with a thiyl radical producing a disulfide radical anion (**reaction 13**), and further electron transfer from that radical anion to $O_2$, forming a disulfide (RSSR) and a superoxide radical anion ($O_2^{\bullet -}$) (**reaction 14**) (according to Clare et al., Biochim. Biophys. Acta 1504: 196–219, 2001).

***1.1.1.2 Thiyl Radicals*** Thiyl radicals ($RS^{\bullet}$) are generated by either hydrogen abstraction from a free thiol group or by cleavage of disulfide linkages. The latter reaction can occur photolytically (Fig. 1.4, reaction 10) and by addition of an electron (reduction), followed by rapid fragmentation of the radical anion (Fig. 1.4, reaction 11) (77). Thiyl radicals react rapidly, but reversibly, with $O_2$ to form peroxyl radicals $RSOO^{\bullet}$ (Fig. 1.5, reaction 12); these can isomerize to sulfonyl radicals $RS(=O)O^{\bullet}$ and give rise to oxyacids and sulfinyl ($RSO^{\bullet}$) radicals (78). At physiological pH values, reaction with excess thiol anion ($RS^{-}$) to give a disulfide radical anion (Fig. 1.5, reaction 13) competes with reaction with $O_2$. The disulfide radical anion also reacts readily with $O_2$ via electron transfer to give the disulfide and $O_2^{\bullet -}$ (Fig. 1.5, reaction 14). Thiyl radicals readily dimerize, and thereby give rise to (inter- or intramolecular) protein cross-links, though the occurrence of such reactions may be limited by steric and electronic factors (52).

***1.1.1.3 Aromatic Ring-Derived Radicals*** Reactions with aromatic side chains generally start by addition to the aromatic ring, and the initial adducts may undergo rapid further reactions. In addition, hydrogen abstraction from the aromatic ring and side chain methylene (-CH2-) groups can be seen. 3,4-DOPA (Fig. 1.6, reactions 15 and 16) is formed following the disproportionation of two initial ring-derived radicals in the absence of $O_2$. In this reaction,

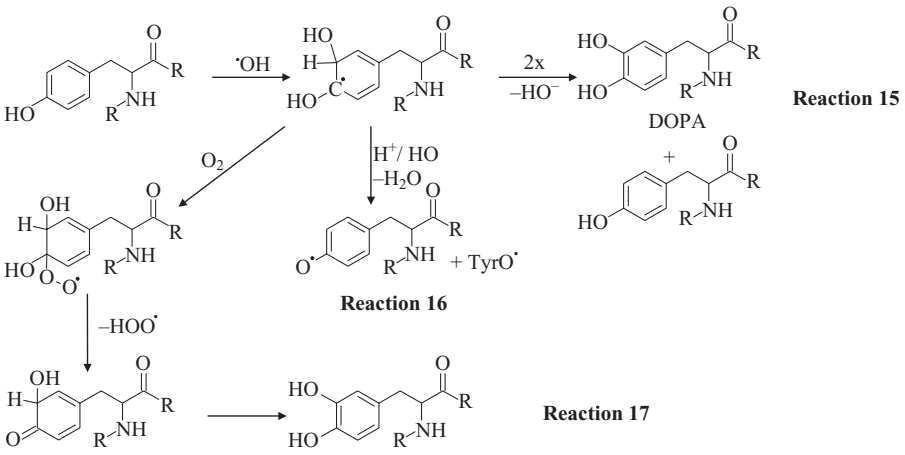



**FIG. 1.6** In reactions with aromatic amino residues, generally additions to the ring occur (first step of reaction 15) and the formed products rapidly undergo further reactions. Oxidation of a tyrosine residue by a hydroxyl radical ($^{\bullet}OH$) forms adduct radicals that can undergo further reactions to form DOPA (3,4-dihydroxyphenylalanine, end products of the **reactions 15** and **17**) of form phenoxyl radicals (**reaction16**). "R" indicates the rest of the protein containing the shown modified side chain (according to Clare et al., Biochim. Biophys. Acta 1504: 196–219, 2001).

$HO^{\bullet}$ reacts with Tyr residues and the formed adduct radicals react further, resulting in DOPA, or rapidly eliminate water, in both acid- and base-catalyzed reactions, to give phenoxyl radicals (Fig. 1.6, reaction 16). In the presence of $O_2$ peroxyl radical formation is followed by rapid elimination of $HOO^{\bullet}$ (Fig. 1.6, reaction 17) and higher yields of DOPA are generated compared with the situation in the absence of $O_2$. This DOPA formation has been used as a tyrosine and protein oxidation marker (79), however this species can also give rise to cellular damage, including DNA damage (80).

Additionally, phenoxyl species are generated by selective oxidants, such as $N_3^{\bullet}$, via one-electron oxidation of the phenolic ring to form a radical cation and subsequent rapid loss of the phenolic proton. They are also generated on a large number of heme, and other proteins, via enzymatic reactions (81, 82). Phenoxyl radicals can dimerize to yield hydroxylated biphenyls (di- or bityrosine; Fig. 1.7, reaction 18), resulting in protein cross-linking. Cross-links between the ortho site and the oxygen atom have also been characterized (Fig. 1.7, reaction 18). Phenoxyl radicals have been implicated in the oxidation of a number of biological targets, including other amino acids, peptides, proteins, lipoproteins, and antioxidants (83, 84).

Similar reactions are observed with Trp, with initial addition occurring to either the benzene ring (Fig. 1.8, reaction 19) or the pyrrole moiety (Fig. 1.8,

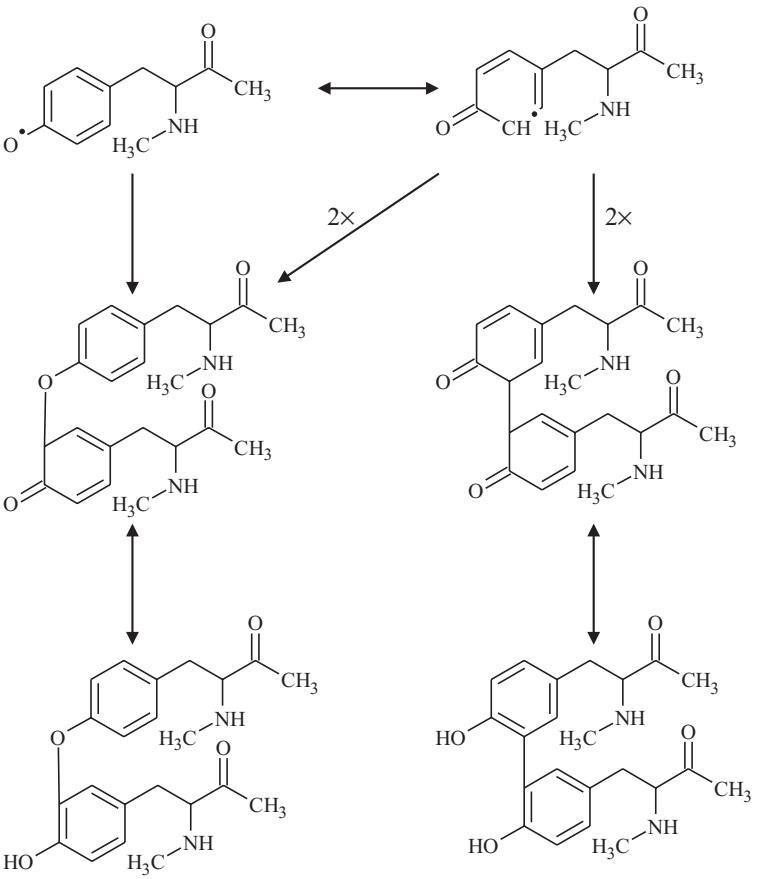



**FIG. 1.7** This figure shows the dimerization of phenoxyl radicals to hydroxylated biphenyls, causing cross-linking of proteins. Both cross-links have been detected (according to Clare et al., Biochim. Biophys. Acta 1504: 196–219, 2001).

reaction 20) (85). In the absence of $O_2$, the benzene ring-derived radicals give either low yields of 4-, 5-, 6-, and 7-hydroxytryptophans or lose water to give the neutral indolyl radical (Fig. 1.8, reaction 21) (86). Indolyl radicals react slowly with $O_2$ (87), but react rapidly with $O_2^{\bullet-}$ to give a hydroperoxide (Fig. 1.8, reaction 22) (88). The remaining benzene ring-derived radicals react with $O_2$ to form peroxyl radicals, some 30% of which eliminate $HOO^{\bullet}/O_2^{\bullet-}$ to give hydroxylated products. The peroxyl radicals formed on reaction of the initial C3 pyrrole ring-derived radical with $O_2$ undergo a ring-opening reaction to give N-formylkynurenine (Fig. 1.8, reaction 23). The formation of hydroxylated products and N-formylkynurenine, and the loss of fluorescence from the parent amino acid, have been employed as markers of Trp oxidation (79).

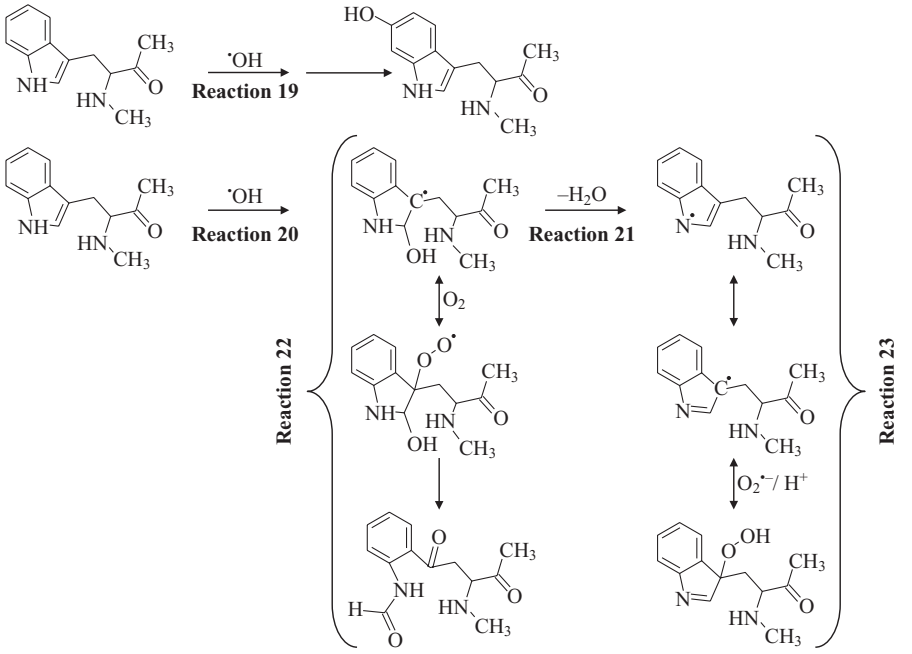



**FIG. 1.8** Reactions of tryptophan with hydroxyl radicals result in an initial addition to the benzene ring (**reaction 19**) or to the pyrole moiety (**reaction 20**). In the absence of $O_2$, the radicals derived from the benzene ring form low amounts of 4-, 5-, 6-, and 7-hydroxytryptophans (**reaction 20**) or the neutral indolyl radical (**reaction 21**). With a slow rate, indolyl radicals react with $O_2$, and with a very fast rate with superoxide ($O_2^{\bullet -}$), forming a hydroperoxide (**reaction 23**). The peroxyl radicals from the reaction with the initial C3 pyrrole ring (from **reaction 20**) can react with $O_2$, resulting in N-formylkynurenine after a ring opening (**reaction 22**) (according to Clare et al., Biochim. Biophys. Acta 1504: 196–219, 2001).

Electron transfer reactions resulting in the formation of radical anions and radical cations are also common with aromatic side chains. Thus, the reaction of the solvated electron with Phe generates a transient radical anion which rapidly protonates to give a cyclohexadienyl radical (Fig. 1.9, reaction 24). Ring radical cations are generated with all the aromatic amino acids on reaction with powerful oxidants, such as $SO_4^{\bullet -}$ (Fig. 1.9, reaction 25), and on direct photoionization (89). The charge of these species is rapidly lost by a number of processes, including hydration (thereby yielding hydroxylated products) and loss of a proton from an adjacent C-H (Phe), N-H (with His or Trp), or O-H bond (with Tyr) (90).

***1.1.1.4 Transfer between Sites*** Transfer reactions between side chains and from side chain to backbone and vice versa may occur. Several transfer

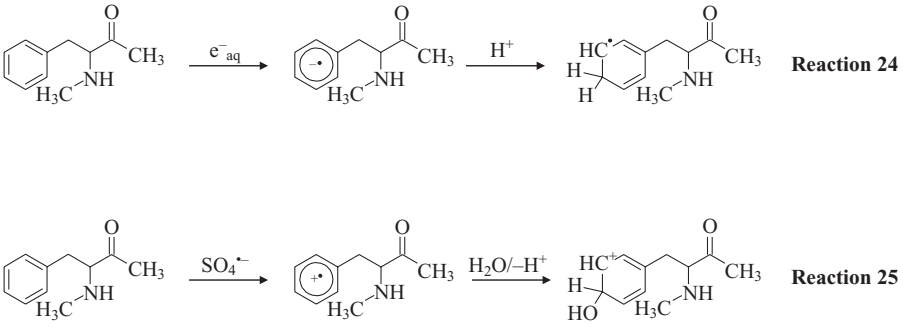



**FIG. 1.9** Reaction of a solvated electron with phenylalanine followed by protonation forms a cyclohexadienyl radical (**reaction 24**). Strong oxidants like $SO_4^{\bullet-}$ or direct photoionization are able to induce ring radical cations at all aromatic amino acids (**reaction 25**) (according to Clare et al., Biochim. Biophys. Acta 1504: 196–219, 2001).

reactions between aromatic side chain-derived radicals have been defined. The reduction potentials of peptide radicals suggest that the ultimate source for oxidizing equivalents is likely to be Tyr residues (or Trp in the absence of these side chains). Thus, peptide radicals are able to oxidize Tyr residues via the formation of the ring radical cation, and subsequent deprotonation to give the phenoxyl radical. These reactions are in equilibrium, so Tyr phenoxyl radicals can be repaired by high concentrations of thiols such as Cys, yielding thiyl radicals. This process is enhanced by excess of thiol anions, as the thiyl radicals generated are removed via the formation of the disulfide radical anion.

Reaction of Trp with $N_3^{\bullet}$ results in the generation of the neutral indolyl radical. If such species are generated on peptides or proteins that also contain Tyr residues, rapid oxidation of the latter residues to give phenoxyl radicals is observed via electron transfer (91). This type of transfer process has been investigated in a 62-amino-acid peptide (erabutoxin B) that contains single Trp (Trp-25) and Tyr (Tyr-29) residues. Slow transfer is observed in this case; this is attributed to the rigid nature of this peptide that contains four disulfide bonds (92). This study suggests that rapid electron transfer requires either direct contact of the reactive residues or contact via suitable intermediate species, and that the peptide backbone does not provide a transfer pathway.

Disulfide bonds (cystine residues) can act as a major source for electrons arising from electron transfer by reducing species. Thus, initial addition of solvated electrons to both the backbone carbonyl groups of peptide bonds and at some side chain sites (e.g., aromatic residues) can result in the ultimate reduction of cystine groups. The yield of initial electrons that end up at disulfide sites depends on the protein; with lysozyme, it is nearly 65%, whereas with RNase A, it is nearly 20%. The latter observation is of particular interest as the disulfide groups in this protein are internalized and inaccessible to species

in bulk solution. Transfer occurs via hydrogen bonding networks, with the backbone acting as an efficient conduit, unlike the oxidative pathway (93). Information on the rates and pathways of transfer cannot be readily obtained in many of these systems due to the random nature of the initial electron addition. Studies with modified metalloproteins have, however, provided information about the mechanisms and control of electron transfer within proteins (94, 95).

Only the transfer to the most readily oxidized side chains (aromatic, Cys, and cystine) is observed in the transfer between side chain and backbone. Reaction of α-carbon radicals with cystine occurs by homolytic substitution to give cross-linked thioethers (96).

Backbone to side chain transfers can occur readily when the radical is centered on other sites apart from the α-carbon. Thus (nitrogen-centered) amidyl radicals generated by photolysis of N-haloamino acid derivatives (e.g., those formed on reaction of HOCl with backbone amide groups) readily abstract hydrogen atoms from side chain sites, with intramolecular 1,5-hydrogen abstraction being particularly rapid. Abstraction of side-chain hydrogen atoms has also been shown to occur with excited-state carbonyl functions on the backbone, particularly when geometrical restraints prevent intramolecular reactions to give α-carbon-centered radicals; these reactions can also occur with excited carbonyl functions on side chains (97). While 1,5- and 1,6-hydrogen atom transfer reactions are not unusual, the efficiency of intramolecular hydrogen abstraction decreases as the transition state ring size increases. Larger transition states have been invoked to explain some long-range photochemically induced transfer reactions of oligopeptide-linked anthraquinones. These reactions involve 1,19- and 1,21-hydrogen atom transfer, and are highly regioselective for coupling of the α-carbon of a Gly residue to a specific carbonyl group on the anthraquinone (98).

Hydrogen abstraction at the γ-carbon position on side chains can yield dehydropeptides via peroxyl radical formation (63). The dehydropeptides undergo base hydrolysis to give a new amide function and a keto acid. Thus, initial side-chain damage can result in backbone cleavage via the intermediacy of a peroxyl species. The three-dimensional structure of a peptide can also affect the chemistry of side chain-derived peroxyl radicals (46).

Alkoxyl radicals formed at C3 on peptides and proteins undergo L-scission reactions to give α-carbon species (99). Alkoxyl radicals formed on Ala side chains readily lose formaldehyde to generate the corresponding Gly α-carbon species (Fig. 1.10, reaction 26). This process predominates over other reactions of such alkoxyl radicals (e.g., 1,2-hydrogen shifts) due to the stability of the α-carbon radical, and appears to occur with a range of C3 side-chain alkoxyl radicals.

### 1.1.2 Reactive Compounds Mediating in Protein Oxidation

Metabolically, oxygen can be incorporated into amino acids by many reactions using a large variety of organic molecules and such abundant molecules as

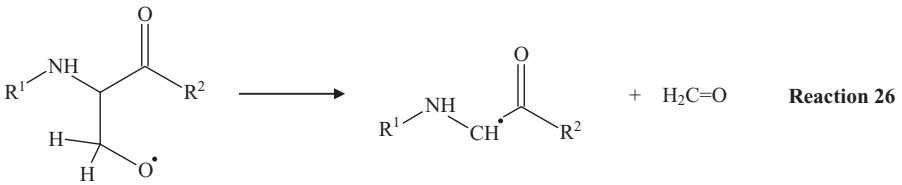



**FIG. 1.10** An alkoxyl radical formed on alanine side chains releases formaldehyde while generating the corresponding glycine α-carbon species (**reaction 26**) (according to Clare et al., Biochim. Biophys. Acta 1504: 196–219, 2001).

**Fenton chemistry:**

$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$ **Reaction 27**

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$ **Reaction 28**

**Haber–Weiss reaction:**

$O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^-$ **Reaction 29**

**FIG. 1.11** In this figure, Fenton chemistry is shown as the linked reduction of $Fe^{3+}$ to $Fe^{2+}$ in the reducing environment of a cell catalyzing the continuous formation of hydroxyl radicals ($^{\bullet}OH$) from $H_2O_2$ (**reactions 27** and **28**). The Haber–Weiss reaction (**reaction 29**) describes the formation of hydroxyl radicals from $H_2O_2$ in the presence of an adequate electron donator (in this case, the superoxide radical anion, $O_2^{\bullet -}$).

water. Indeed, amino acid biosynthesis rarely uses $O_2$ directly, an exception being the synthesis of tyrosine from phenylalanine (1).

Free radicals are chemicals with unpaired electrons in their outer orbitals. Free radicals have different reactivities, ranging from the high reactivity of the hydroxyl radical to the low reactivity of melanins. Superoxide anion and nitric oxide are believed to be produced continuously in aerobic cells, the superoxide preferentially in the mitochondria. Superoxide anion is dismutated to hydrogen peroxide by the Mn-SOD located in the matrix of mitochondria. Superoxide and $H_2O_2$ are able to initiate Fenton or Haber–Weiss chemical reaction and $^{\bullet}OH$ formation (Fig. 1.11). This reaction is catalyzed by the $Fe^{2+}$ ion (100).

Radical-mediated damage to proteins may be initiated by electron leakage, metal ion-dependent reactions, and autoxidation of lipids and sugars. The consequent protein oxidation is $O_2^{\bullet -}$ dependent and involves several propagating radicals, notably alkoxyl radicals. Its products include several categories of reactive species and a range of stable products whose chemistry is currently being elucidated (44).

Two categories of reactive, but nonradical, intermediates in protein oxidation have been identified. Reductive moieties, notably DOPA formed from

tyrosine, can reduce transition metal ions, thus enhancing reactions with hydroperoxides, and are also able to induce radical formation in reactions with $O_2$ (101–102). The other category are the hydroperoxides formed particularly on aliphatic side chains, but probably also on main-chain α-carbons (72). These can be decomposed by transition metal ions to give further radicals, which may propagate reaction chains. The hydroperoxides may also be reductively detoxified to hydroxides, probably without radical formation (103).

3-NY formation involves reactive nitrogen intermediates. Nitric oxide, peroxynitrite, nitrite, and reactions between hypochlorite and nitrogen-containing compounds are the sources of many more intermediates. Several of these species can also give rise to both hydroxylated aromatic residues and tyrosyl (phenoxyl) radicals, and hence dityrosine. Dityrosine can also be formed by the myeloperoxidase (MPO)/chloride/$H_2O_2$ system, with either protein-bound or free tyrosine, as judged by model experiments. Whether this also plays a major role *in vivo* is not clear, since the dityrosine formation might be paralleled by 3-chlorotyrosine formation. This is complicated by the possible further oxidation of dityrosine (104).

***1.1.2.1 Hydroxyl Radical*** During the oxidation of aliphatic amino acids by $HO^{\bullet}$, hydroxylated derivatives, notably of the side chains, are formed. These were partially characterized by Kopoldova and coworkers (105), and have been fully designed for valine and leucine (103). During the oxidation of aromatic residues, the formation of phenoxyl radicals from tyrosine and their conversion into dityrosine and further products can occur, especially if there are no reductants to repair the tyrosyl radicals (e.g., thiols, vitamin E) and if there are vicinal tyrosyl radicals (106). Hydroxylation of phenylalanine, tyrosine, and tryptophan is also one of the characteristic reactions of hydroxyl radicals, and similar reactions of histidine (giving 2-oxohistidine) are important (58). *In vitro* studies demonstrate that the hydroxyl radical converts L-phenylalanine into M-tyrosine, an unnatural isomer of L-tyrosine (107).

The highly reactive $^{\bullet}OH$ radicals are also able to form protein radicals of various life spans. The involvement of such radicals in biological catalysis was suggested earlier (108). The electron desaturation of proteins can be produced by $^{\bullet}OH$ radicals so that the practically continuous depletion of electrons by $^{\bullet}OH$ radicals may represent the mechanisms involved in the overcome of the energetic barrier between the valency and the conductive bands of the proteins (109). $^{\bullet}OH$ radicals formed by the Fenton reaction are able to attack practically all amino acids and proteins even under mild chemical conditions (110). Therefore, it was shown that the reaction of $^{\bullet}OH$ with free Gly can give rise to nitrogen-centered radicals as a result of one-electron oxidation of the free amine group to give an aminium radical cation ($RNH_2^{+\bullet}$) or the neutral aminyl radical ($RNH^{\bullet}$) (111).

Reactions of radicals such as $^{\bullet}OH$ with His are complex, with initial addition occurring at C2, C4, and C5 of the imidazole ring (Fig. 1.12). These radicals can react with $O_2$ to give peroxyl radicals or undergo base-catalyzed loss of

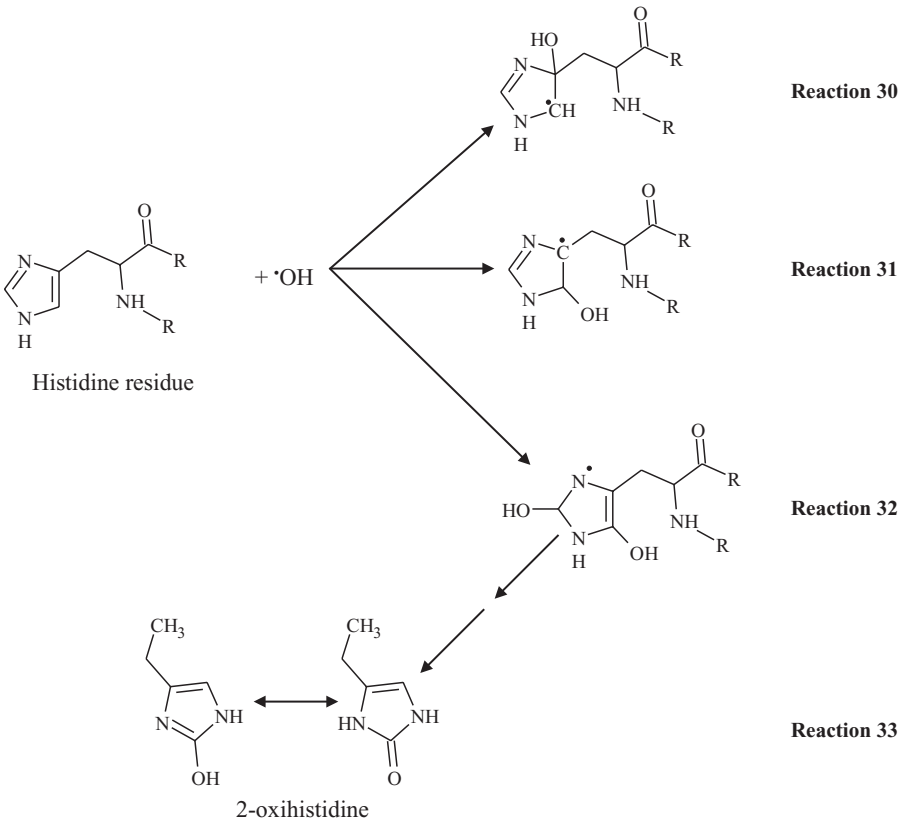



**FIG. 1.12** Different aducts from the reaction of a histidine residue with the hydroxyl radical at different C-atoms from the imidazole ring (**reactions 30–32**). After several intermediate steps, 2-oxihistidine can be one of the final products occurring (**reaction 33**) (according to Clare et al., Biochim. Biophys. Acta 1504: 196–219, 2001).

water to give a stabilized diazacyclopentadienyl radical. The final products of these reactions have not been completely characterized, but include 2-oxo-histidine, asparagine, aspartic acid, hydroxylated derivatives, and hydroperoxides (58).

***1.1.2.2 Superoxide Radicals*** The superoxide anion is formed by the univalent reduction of triplet-state molecular oxygen ($^3O_2$). This process is mediated by enzymes such as NAD(P)H oxidases and xanthine oxidase or nonenzymatically by redox-reactive compounds such as the semiubiquinone compound of the mitochondrial electron transport chain. SODs convert superoxide enzymatically into hydrogen peroxide. In biological tissues, superoxide can also be converted nonenzymatically into the nonradical species hydrogen peroxide and singlet oxygen ($^1O_2$) (112).

The primary free radical in most oxygenated biological systems is the superoxide radical ($O_2^{\bullet-}$), which is in equilibrium with its protonated form, the hydroperoxyl radical ($HO_2^{\bullet}$) (113). The major sources of these radicals are modest leakages from the electron transport chains of mitochondria, chloroplasts, and endoplasmic reticulum (ER). Although $O_2^{\bullet-}$ is relatively unreactive in comparison with many other radicals, biological systems can convert it into other more reactive species, such as peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$), and hydroxyl ($^{\bullet}OH$) radicals. The last of these can originate from the Fenton reaction, in which the metal ion redox cycles, with reduction effected by $O_2^{\bullet-}$ and oxidation effected by its dismutation product, hydrogen peroxide ($H_2O_2$). Iron and copper are biologically important transition metal ions, with their reduced forms capable of rapidly cleaving organic (including lipid) hydroperoxides, forming radicals that can initiate chain reactions, ultimately giving stable products such as lipid hydroxides (44).

The two-electron (nonradical) oxidant hypochlorite is a major product of stimulated neutrophils, which produce superoxide radicals which dismute to $H_2O_2$ and then convert it into hypohalous acids by the action of MPO in the presence of halides. Although the nonradical nature of this oxidant makes it chemically distinctive, its occurrence in biological systems is an important process for the organism and was reviewed in Reference 44.

Of the radicals formed in biological systems, the greatest attention has been focused on superoxide, the species formed when oxygen is reduced by a single electron (Fig. 1.13, reaction 34) (114). Superoxide undergoes a dismutation to form hydrogen peroxide (Fig. 1.13, reaction 35), therefore $H_2O_2$ is also generally present in superoxide-generating systems. In addition, superoxide can be protonated to form the hydroperoxyl radical (Fig. 1.13, reaction 36) (114).

As mentioned, superoxide is found to be formed in all aerobically metabolizing cells. For example, electrons that appear to "leak" out of the mitochondrial respiratory chain are transferred to oxygen and generate superoxide; these radicals may cause cooxidation of xenobiotics and/or initiate pathological changes. In addition, macrophages and certain other phagocytic cells produce superoxide during the oxidative burst that follows their activation.

| | |
|---|---|
| $O_2 + e^- \rightarrow O_2^{\bullet-}$ | **Reaction 34** |
| $2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$ | **Reaction 35** |
| $2\,O_2^{\bullet-} + H^+ \rightarrow HOO^{\bullet}$ | **Reaction 36** |

**FIG. 1.13** Superoxide ($O_2^{\bullet-}$) is the main primary radical formed in cellular systems by oxygen reduction by a single electron (**reaction 34**). Enzymes like SOD can form hydroperoxide ($H_2O_2$) by addition of two protons. In an acidic/neutral environment, superoxide can be protonated and forms the electroneutral hydroperoxyl radical ($HOO^{\bullet}$) (**reaction 36**) (according to Pryor, Annu. Rev. Physiol. 48: 657–667, 1986).

Superoxide is formed when electronegative compounds intercept electrons from normal cellular electron transport and then reduce oxygen, a process called redox cycling.

It has become clear that superoxide is produced during the reperfusion of oxygenated blood into tissue that has briefly been anoxic (115–116). It was observed that organs can be maintained in an anoxic state for some time with little or no damage; however, when arteries are unclamped/reopened and aerated blood is allowed to reperfuse the organ, tissue damage can be induced suddenly and severely by oxidative damage. A number of investigators have discovered that this damage can be mitigated or prevented if SOD or other protective species (antioxidants) are added to the blood during reperfusion.

By comparing the superoxide anion-generating capacity of subcellular fractions from the lungs of neonatal and adult rats, the microsomal fractions from adult rats produced approximately three times more superoxide. This was explained on the basis that microsomes from adult rats was shown to contain almost a threefold greater content of CYP450 and a twofold greater concentration of NADPH cytochrome c reductase (117).

Boveris et al. (118) and Boveris and Chance (119) demonstrated that large amounts of superoxide anion were generated by mitochondria during the process of complex I and II reduction of coenzyme Q10 and its oxidation by complex III. Chance et al. in 1979 (120) estimated that 1–3% of inspired oxygen was converted to superoxide anions; such large amounts of product would indeed be potentially highly toxic to cells. Coenzyme Q10 is known to occur in all subcellular membranes and has a functional role in many known membrane oxidoreductase systems localized therein, notably in the mitochondria, plasmalemma, the Golgi apparatus (121), and lysosomes (122). Coenzyme Q10 oxidoreductase systems play a major role in the regulation of subcellular metabolism through the agency of superoxide anion formation and metabolome modulation. The global functions of coenzyme Q10 in relation to subcellular bioenergy systems, redox equilibrium, metabolic flux modulation, gene regulation, and oxygen radical formation are referred to in studies (123).

The activity of the important nuclear transcription factor nuclear factor κB (NFκB) is regulated by superoxide anion formation. NFκB is maintained in the cytosol in an inactive form bound to the inhibitor IκBα. Following plasma membrane superoxide and $H_2O_2$ formation, induced by various cell effectors (e.g., cytokines, hormones) and regulated by Ras 1 (G protein), a transduction protein phosphokinase acts to phosphorylate IκBα. This phosphorylation results in dissociation of the complex IκBα-P ubiquitination and its destruction by the proteasome, and release of NFκB to translocate to the nucleus and function as a major transcription regulator (124).

Protective responses against superoxide are controlled by the sox locus (125), which regulates the induction of ~10 proteins, including Mn-SOD, NADPH : ferredoxin oxidoreductase, and G6PDH (126). One of the gene products from the sox locus, the SoxR protein, exists in solution as a homodimer containing two stable (2Fe-2S) centers that are anchored to four

Cys residues near the COOH terminals (125). Under normal physiological conditions, these iron–sulfur centers are in the reduced state. They are readily oxidized, however, under oxidative stress. The oxidative process is reversible if the oxidative stress conditions are removed. Only the oxidized form of SoxR stimulates transcription of SoxS. Both the oxidized and the reduced forms of SoxR can bind to DNA but interact differently with RNA polymerase (127).

***1.1.2.3 Hydrogen Peroxide*** Hydrogen peroxide, like most peroxides, is very sensitive to decomposition. It is enzymatically decomposed by catalase and GSH peroxidase, and by extremely low concentrations of transition metals such as iron, even if it is chelated in some complexes such as EDTA or adenosine diphosphate (ADP) (114). Therefore, in the presence of reduced transition metals (e.g., ferrous or cuprous ions), hydrogen peroxide can be converted into the highly reactive hydroxyl radical ($^{\bullet}OH$). Alternatively, hydrogen peroxide may be converted into water and $O_2$ by the enzymes catalase (128).

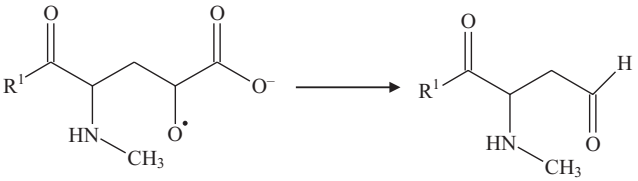
Superoxide anion and hydrogen peroxide formation are essential to normal cellular function; they constitute a second messenger system absolutely required for the regulation of the metabolome. Embraced within this regulation is the modulation of cellular redox status, bioenergy output, gene expression and cell differentiation (123, 129). $H_2O_2$ is also known to play roles in energy metabolism, multisystem redox regulation, protein turnover, and neutrophil/macrophage activation (124).

Since protein tyrosine phosphatases share a common sequence motif with a catalytically essential Cys residue in the active center, this group of enzymes is especially sensitive to hydrogen peroxide (130). Therefore, hydrogen peroxide at concentrations in the order of 1 mM converts this Cys residue into Cys sulfenic acid (Cys-SOH) and thereby inactivates these enzymes.

***1.1.2.4 Lipid Peroxyl Radicals*** The preferred reaction of lipid peroxyl ($LOO^{\bullet}$) radicals is hydrogen abstraction from other molecules, however the reactions are not restricted to these, so lipid peroxyl radicals are also able to produce epoxides at double bonds. In the metabolism, these reactions are probably responsible for the epoxidation of cholesterol (131). Epoxides of cholesterol have been detected in tissue after a myocardial infarction (132) and after burn injury (133), underlining the physiological importance of epoxidation. Interestingly, these products were found to be highly toxic, probably due to their ability to react with nucleophiles, for example, glutathione (GSH) or nucleobases. Concerning protein oxidation, it is known that $LOO^{\bullet}$ radicals are able to remove hydrogens from secondary alcohols or amines within proteins. By this reaction, such compounds are transformed to carbonyl groups, explaining the accumulation of this groups in proteins and nucleic acids in aged tissue (134).

***1.1.2.5 Alkoxyl Radicals*** Alkoxyl radicals apparently have a greater importance in protein oxidation chains (71) than they do in LPO, in which peroxyl radicals are the key chain-propagating species. Alkoxyl radicals can

β-Scission of glutamine




**Reaction 37**

**FIG. 1.14** β-Scission of glutamine, releasing a carboxyl radical ($CO_2^{\bullet-}$) (according to Clare et al., Biochim. Biophys. Acta 1504: 196–219, 2001).

be generated from peroxyl radicals via a tetroxide, or one-electron reduction of alkyl hydroperoxides or dialkylperoxides. Alkoxyl radicals undergo rapid addition and hydrogen abstraction reactions as well as facile unimolecular fragmentation and rearrangement reactions. Most primary and secondary alkoxyl radicals undergo rapid hydrogen shift reactions which result in the formation of α-hydroxyalkyl radicals. These reactions compete with intra- (usually 1,5-hydrogen shifts) and intermolecular hydrogen abstraction processes to give alcohols. β-Fragmentation reactions occur with alkoxyl radicals (Fig. 1.14), where 1,2-hydrogen shift reactions are impossible, and with primary or secondary alkoxyl radicals where a particularly stable carbon-centered radical and aldehyde/ketone are formed. Relief of steric strain also plays a role in determining the extent of these reactions, which can be rapid in aqueous solution (135). These processes can result in the transfer of damage from a side chain to the backbone.

Backbone-derived alkoxyl radicals, formed via cross-termination reactions of peroxyl radicals or decomposition of backbone hydroperoxides, undergo rapid β-scission (fragmentation) reactions. This results in the formation of carbonyl groups and acyl radicals of partial structure $^{\bullet}C(O)NHR$ (see Figs. 1.15 and 1.16, reaction 38). With C-terminal α-carbon alkoxyl radicals, $CO_2^{\bullet-}$ (or $^{\bullet}C(O)NH_2$, in the case of C-terminal amides) is released (72). Analysis of the products obtained from protein cleavage reactions should indicate the percentage of backbone cleavage occurring via the alternative pathways shown in Figure 1.15. Fragments with new N-termini, as opposed to products with blocked N-termini that arise via the imine pathway, have been detected with oxidized proteins; these materials may be alkoxyl radical L-scission products (136). Fragments consistent with the alkoxyl radical pathway have also been detected during backbone cleavage of the R1 subunit of ribonucleotide reductase, where a backbone, Gly-derived, α-carbon radical is involved (137).

***1.1.2.6 $^{\bullet}$NO and Peroxynitrite*** $^{\bullet}$NO is classified as a free radical in terms of its unpaired electron, but since it is not able to initiate typical damage reactions to biomolecules it is instead a nonreactive radical. Nitric oxide is

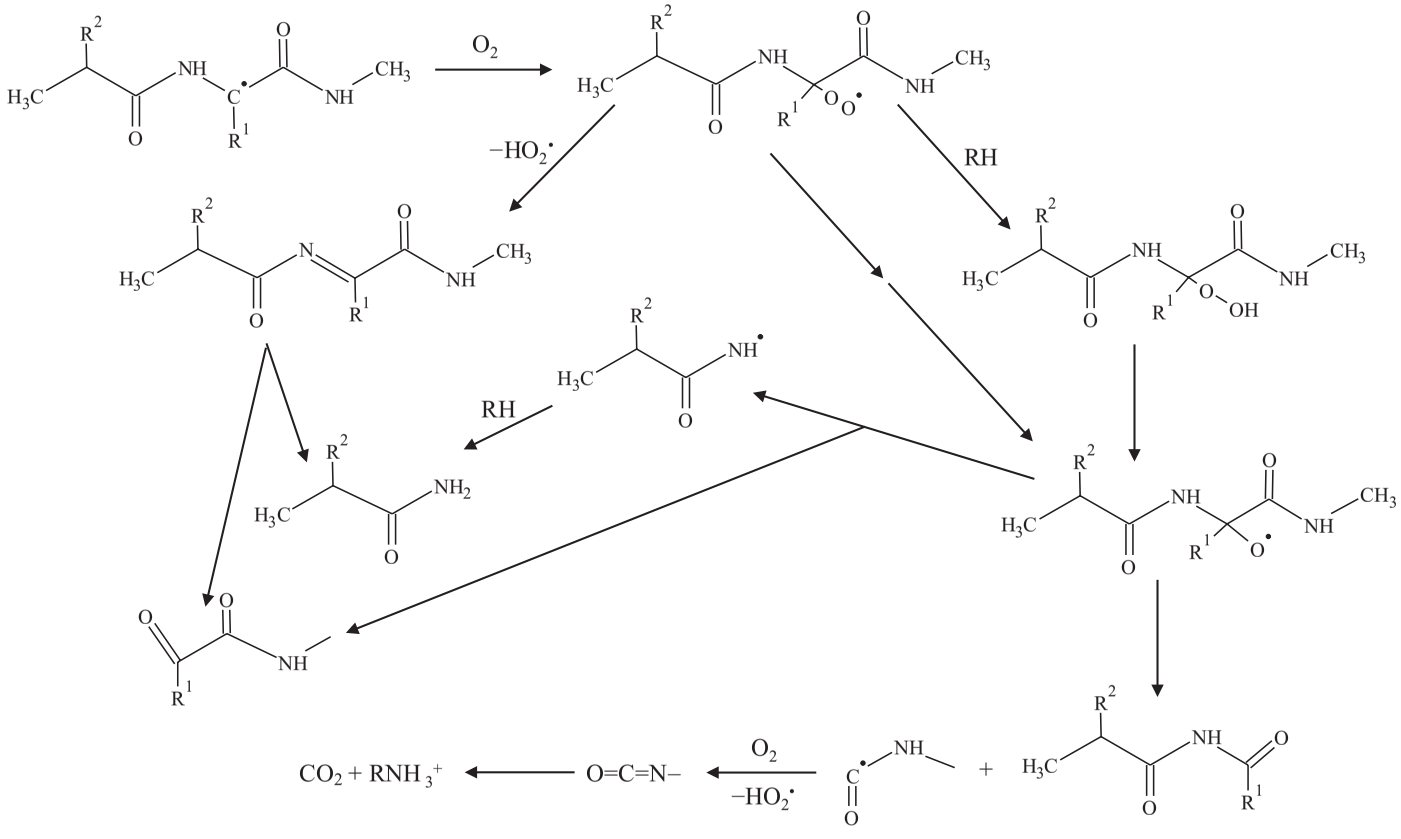



**FIG. 1.15** This scheme shows the possible mechanism behind backbone cleavage in proteins after an initial abstraction of an H-atom from their α-carbon site (according to Clare et al., Biochim. Biophys. Acta 1504: 196–219, 2001).

| | |
|---|---|
| ~C(O)-NH-C(O•)(R)-C(O)-NHR~<br>~C(O)-NH-C(R)=O + •C(O)-NHR~ | **Reaction 38** |

**FIG. 1.16** During cross-termination, reactions of peroxyl radicals or the decomposition of backbone hydroperoxides, backbone-derived alkoxyl radicals, can be formed that undergo rapid β-scission (fragmentation) reactions. In this case, carbonyl groups and acyl radicals of the structure •C(O)NHR are formed (according to Clare et al., Biochim. Biophys. Acta 1504: 196–219, 2001).

produced *in vivo* during the oxidation of one of the terminal guanido-nitrogen atoms of L-arginine (138) to L-citrulline catalyzed by •NO synthase (NOS), in the presence of NADPH and $O_2$ (139, 140).

•NO is well recognized as a signaling molecule regulating blood vessel dilation, acting as a neurotransmitter and metabolic cell regulator, as well as in an increasing number of other physiological reactions such as hemoglobin allosteric modulation, $O_2$ transport regulation, cellular energy control, mitochondrial proliferation, and Fe homeostasis (124). Depending on the microenvironment, •NO can be converted to various other RNS such as nitrosonium cation ($^{\bullet}NO^{+}$), nitroxyl anion ($^{\bullet}NO^{-}$), or peroxynitrite ($ONOO^{-}$) (141).

Peroxynitrite ($ONOO^{-}$) is a highly toxic nonradical oxidant arising from the reaction between superoxide anion and •NO (124). This reactive molecule considered to be an RNS is able to nitrosate Cys sulfhydryl groups (142), nitrate tyrosine, or tryptophan residues of proteins, and oxidize methionine residues to methionine sulfoxide (3). Interestingly, the ability to mediate such reactions is strongly influenced by the availability of carbon dioxide. This is due to the fact that peroxynitrite reacts rapidly with $CO_2$, forming nitrosoperoxycarbonate ($ONOOCO_2^{-}$) (143). Therefore, the oxidation of proteinogenic methionine side chains by peroxynitrite is prevented by physiological concentrations of $CO_2$. Furthermore, the nitration of tyrosine residues is almost completely dependent upon the presence of $CO_2$. The importance of tyrosine nitration in the metabolism is underscored by the demonstration that tyrosine nitration prevents the phosphorylation or adenylation of tyrosine residues of regulatory proteins. However, in some cases, such a nitration can lead to these enzymatic posttranslational modifications (3). Following the reaction of peroxynitrite with tyrosine in proteins, 3-NY is formed. Furthermore, $NO_2^{\bullet}$ is also able to oxidize tyrosine to 3-NY (144, 145). Moreover, $NO_2^{-}$ is a substrate for mammalian peroxidases and forms $NO_2^{\bullet}$ via peroxidase-catalyzed oxidation of $NO_2^{-}$. This provides an additional pathway for the formation of 3-NY (146). In contrast, Inoue et al. have described the competition of •NO and molecular oxygen ($O_2$) at the active center of cytochrome oxidase, the biological reduction of $O_2$ to $O_2^{\bullet-}$, and the interaction of •NO and $O_2^{\bullet-}$, a reaction that annihilates the two free radicals, as a "cross-talk" between the chemical species as a basic regulatory function in aerobic life (147). It has

been reported that $ONOO^-$ is an enzyme activator, which activates mainly $Ca^{2+}$-dependent ATPase of the ER, itself a major regulator of second messenger $Ca^{2+}$ (148).

Some of the physiological effects of RNS may be mediated through the intermediate formation of S-nitroso-Cys or S-nitroso-glutathione (149). Many of the enzymes that utilize a heme prosthetic group in catalysis are inactivated by •NO. This applies to the heme-containing CYP450-related enzyme NOS, leading to feedback inhibition of •NO production by •NO. The inhibition of NOS was shown to involve the formation of a ferrous-nitrosyl complex (150). Guanylate cyclase belongs to the family of heterodimeric heme proteins and catalyzes the formation of cyclic guanosine monophosphate (cGMP), which is utilized as an intracellular amplifier and second messenger in a large range of physiological responses. •NO binds to the heme moiety of guanylate cyclase, disrupting the planar form of the heme iron. The resulting conformational change activates the enzyme. Its product cGMP modulates the function of protein kinases, phosphodiesterases, ion channels, and other physiologically important targets (128). In vascular endothelial cells, the heme group of guanylate cyclase is among the best-studied molecular targets for •NO (151). •NO binding to guanylate cyclase results in enzyme activation and subsequent conversion of guanosine monophosphate (GMP) to cGMP. The ultimate consequence of cGMP synthesis is relaxation of vascular smooth muscle. Addition of •NO to other heme groups has been reported, and may significantly influence the redox environment of the cell. For instance, Kanner and colleagues (152) have shown that the •NO–myoglobin complex is incapable of catalyzing $H_2O_2$-dependent LPO, although metmyoglobin and oxymyoglobin were able to do so. More recently, •NO has been recognized to form metastable S-nitrosothiol derivatives which may be relevant to signal transduction processes (153). The mechanism of nitrosation is somewhat debatable. Some mechanisms favor indirect pathways, where •NO is first oxidized to the nitrosonium cation ($•NO^+$) or, more likely, to dinitrogen trioxide ($N_2O_3$) before reaction with sulfhydryls. •NO oxidation to $N_2O_2$ requires molecular $O_2$, although other ROS are not involved (154). The full significance of biological S-nitrosation is not clear at this point.

One possible consequence of S-nitrosation is an increase in the effective diffusion distance for •NO. S-nitrosation of hemoglobin may facilitate the delivery of •NO to capillaries determined by perfusion control (155). Another possibility is that S-nitrosation activates (or inhibits) regulatory elements involved in signal transduction cascades in a manner analogous to the reaction of •NO with guanylate cyclase. In support of this latter paradigm, Lander and his colleagues (156) have described S-nitrosation of the monomeric guanosine triphosphate-BiP and oncogene p21-ras. Activation of p21-ras is expected to stimulate downstream protein kinases involved in cell cycle control, apoptosis, and inflammatory gene expression. As expected, •NO activation of p42/p44 mitogen-activated protein kinase (MAPK), c-Jun amino-terminal kinase, and p38-mitogen-actived protein kinase has been reported (157).

In human peripheral blood mononuclear cells and endothelial cells, •NO was found to activate all three MAPK pathways (157, 158). The effect has been attributed to the •NO-mediated stimulation of a membrane-associated protein tyrosine phosphatase activity, which may lead to the dephosphorylation and activation of the Src family protein tyrosine kinase $p56^{lck}$ (159). Another Src family protein kinase, $p60^{c\text{-}src}$, was also found to be activated by •NO in fibroblasts (160). The activation was associated with autophosphorylation at Tyr-416 and S–S bond-mediated aggregation of the kinase molecules. •NO may also activate Ras by S-nitrosylation of Cys-118 (156).

The erosion of telomeric DNA leads to the loss of telomere capping function and the onset of cellular senescence (161). In many actively proliferating cells, telomere erosion is counteracted by a specialized reverse transcriptase called telomerase (162). This enzyme catalyzes the addition of TTAGGG repeats to the 3′ end of telomeric DNA and delays or prevents altogether the onset of senescence. One study has reported that telomerase is upregulated by nitric oxide (•NO), which delayed senescence (163).

Nitric oxide production is localized to neurons, astrocytes, as well as microglia and endothelial cells (164), and has been shown to produce neurotoxicity in the brain (165). It is clear that the cellular context of oligodendrocytes in the brain includes several potential sources of nitric oxide that might generate this gas in response to a variety of stimuli. Rosenberg et al. (166) showed that the toxicity of nitric oxide to oligodendrocytes was not blocked by extracellular SOD or catalase. Moreover, they also caused Cys deprivation of oligodendrocytes and observed the protective action of nitric oxide donors against Cys deprivation-evoked toxicity. Given the important effects of nitric oxide on iron metabolism and iron reactivity, and that Cys deprivation-induced toxicity in oligodendrocytes is blocked by chelation of iron (167), it seems quite plausible that the protective effect of nitric oxide against Cys deprivation-induced toxicity is due to interaction with free or protein-bound endogenous iron. In addition to that, •NO has been reported to promote synaptic plasticity changes such as long-term potentiation in the hippocampus and long-term depression in the cerebellum, as well as learning and memory (168). These actions are primarily mediated via a cGMP-mediated potentiation of glutamate (Glu) release (169). In a study, the major intracellular target of •NO is also found to be the soluble guanylate cyclase (170). For the •NO cytotoxicity in neurodegenerative disorders such as AD and Parkinson's disease, the favored hypothesis implies the formation of peroxynitrite ($ONOO^-$) with subsequent protein nitration and/or nitrosation (171).

From a biological point of view, •NO has dual effects. On one hand, as a signaling molecule and at low concentrations (10–150 nM), •NO has physiological functions as an intracellular and intercellular regulatory messenger. On the other hand, •NO behaves as a cytotoxic molecule at high levels (>300 nM) in pathological and pathophysiological situations (172). Several studies have shown the protective effects of nitric oxide in a variety of paradigms of cell injury and cell death. These include direct scavenging of free radicals (173);

effects on intracellular iron metabolism, including interaction with iron to form nitrosyl-iron complexes (174), preventing release of iron from ferritin (175), or stimulating iron-responsive RNA binding elements such as *cis*-aconitase (176); inactivation of caspases (177); activation of a cGMP-dependent survival pathway, as has been seen in PC12 cells (178) and tumor necrosis factor-α (TNF-α)-induced apoptosis in endothelial cells (179); inducing expression of protective proteins such as heat shock proteins (HSPs) (180); inhibition of NFκB activation (181); inhibition of glyceraldehyde-3-phosphate dehydrogenase (182), whose activity appears to be required in one paradigm of neuronal apoptosis (183); and oxidation in neurons of a redox modulatory site on the N-methyl-D-aspartic acetate (NMDA) receptor, resulting in a decrease in NMDA receptor-mediated currents (184), a mechanism that remains controversial (185).

In the heart, an •NO donor or eNOS knockout was reducing neutrophil-mediated injury by lowering neutrophil adhesion and, therefore, preservation of endothelial function (186). Therefore, eNOS-dependent •NO synthesis regulates arterial pressure, however this regulation was proposed to be malfunctioning in human hypertension (187).

Interestingly, nitric oxide is an inhibitor of the mitochondrial electron transport (188), binding reversibly to cytochrome oxidase. Additionally, the mitochondrial aconitase is a preferred target of superoxide and peroxynitrite. In particular, the [4Fe-4S] cluster might be disrupted, leading to a loss of enzymatic function (189).

Because of the relatively short half-life and low levels of •NO in tissues and biological fluids, measurements of •NO production are often performed by determination of nitrite and nitrate—the final products of •NO metabolism. These metabolites are either partly formed by autooxidation of nitric oxide or are derived from the diet. Toprakci et al. found that •NO release declines with age in healthy people (190). This reduction in •NO synthesis with age is perhaps a component of the vascular diseases with increasing age. Nitrite ($NO_2^-$), the more toxic metabolite, can be formed from nitrate ($NO_3^-$). Nitrate is also taken up by the diet, so it was concluded that eating of preserved meats could increase the risk of bowel cancer by 50% due to the enhanced nitrate consumption (191).

As previously mentioned, •NO is derived from the reduced NADPH-dependent oxidation of L-arginine by the enzyme NOS. A constitutively expressed version of NOS exists in endothelium and neurons. These isoforms are transiently activated by $Ca^{2+}$ influx into the cytosolic compartment, and they synthesize •NO in brief pulses of activity. •NO diffuses freely across cell membranes until binding a suitable target.

***1.1.2.7 Hypochlorous Acid*** Hypochlorous acid/hypochlorite is regarded as one of the main bactericidal factors *in vivo*, and is therefore an important protector against pathogens during immune response (192). However, numerous evidence that its excessive formation may lead to host tissue injuries have

been accumulated. Products of hypochlorous acid/hypochlorite reactions with biological compounds have been found in many disorders (193). The main markers of the presence of hypochlorous acid/hypochlorite are chlorinated tyrosine (3- and 3,5-dichlorotyrosine) (194). N-chloroamino acids are unstable products of reactions of hypochlorous acid/hypochlorite. Their formation is generally considered as a protective reaction against the toxicity of hypochlorite/hypochlorous acid; in contrast, they may propagate the reactions of oxidation and chlorination reactions, being second messengers of the toxicity of hypochlorous acid/hypochlorite. In proteins, Cys and Met are preferentially attacked by N-chloroamino acids (195), although at rates lower by several orders of magnitude than those for $HOCl/OCl^-$ (196).

Except for covalent cross-links, noncovalent protein aggregates can be formed in proteins treated with $HOCl/OCl^-$ as a consequence of alterations in polypeptide chain folding, and of exposure of hydrophobic regions, leading to aggregation (197). Proteins exposed to $HOCl/OCl^-$ have been documented to undergo fragmentation as an end result of radical formation from lysine side-chain residues (69). It has also been noticed that the polypeptide backbone cleavage induced by $HOCl/OCl^-$ may be increased in the presence of superoxide anion, which accelerates the breakdown of the nitrogen–chloride bond in N-chlorolysine and increases the amount of lysine-derived radicals (198). It has been documented previously that protein exposure to $HOCl/OCl^-$ results in a decrease of the tryptophan content. Incubation of low-density lipoprotein (LDL) with $HOCl/OCl^-$ resulted in a more than 35% tryptophan loss and complete Cys oxidation in apolipoprotein B (199). The tryptophan loss induced by the chlorocompounds was accompanied by formylkynurenine formation, most efficient for $HOCl/OCl^-$ and hydrophobic N-chloroamino acids, N-chloroaspartic acid being completely ineffective. It has been demonstrated that the indole ring of tryptophan may undergo either chlorination or oxidation by $HOCl/OCl^-$, depending on the neighbor amino acids which gives rise to various products of tryptophan modifications (200).

Reaction of hypochlorous acid (HOCl) with proteins can result in the formation of unstable chloramines (RNHCl) or chloramides (R-C(O)-NCl-R′) via reaction with amines and amides. Decomposition of these species can give nitrogen-centered radicals via cleavage of the N–Cl bond. These nitrogen-centered radicals undergo various rearrangement reactions to give carbon-centered radicals via mechanisms analogous to those outlined above for alkoxyl radicals (69). Reaction of HOCl with proteins can result in backbone cleavage. This process may involve nitrogen-centered radicals formed on decomposition of intermediate chloramide species or nonradical dehydrochlorination and hydrolysis of the imines (69).

### 1.1.3 Enzymatic Systems Playing a Role in Protein Oxidation

Enzymes are among the major sources generating ROS/RNS, which are important in protein oxidation processes, and it is known that in the catalytic

process, a number of enzymes use radical species for enzymatic reactions (201). These radicals are located in tyrosines, tryptophans, glycines, or thiols. Ribonucleotide reductase is a good example of an enzyme which contains a tyrosyl radical and probably also a thiyl radical in order to abstract a hydrogen atom from the ribose ring of the substrate (202). The reaction of these protein radicals with $O_2$ (or possibly other species) can cause enzyme inactivation by backbone cleavage rather than side-chain alterations. Another example is the *Escherichia coli* pyruvate formate lyase, which contains an intrinsic glycyl radical and is inactivated in the presence of $O_2$ or hypophosphite. The damage in the enzyme is limited and thus is reversible (203). Reversal may be provided by the loss of $O_2$ from $ROO^{\bullet}$ and the resulting stable carbon-centered species.

Many oxidative enzymes, such as LOXs and the CYP450 family, can generate radical species during their interaction with substrates and, in some cases, inactivate themselves via this way. Thus, lipid peroxyl radicals are released during lipoxygenase action, and the product hydroperoxides of linoleic acid can inactivate the enzyme if iron is available (from the enzyme or elsewhere). Inactivation is enhanced by $O_2$, and such reactions give rise to the "hydroperoxidase" activity of this enzyme (204).

***1.1.3.1 NADPH Oxidase*** NADPH oxidase (NOX) is a multiple subunit electron transport system which was discovered in neutrophils, where it catalyzes one electron reduction of oxygen to produce $O_2^{\bullet-}$, using NADPH as the electron donor during phagocytosis and plays a role in immune protection with its bactericidal activity (205–207). This enzymatic system plays a key role in generating ROS in fibroblasts, vascular smooth muscle cells (VSMCs), and endothelial cells besides phagocytic cells. The NOX subunits are shown to be present in human blood vessels, including atherosclerotic coronary arteries, in veins, and mammary arteries with coronary artery disease, which strengthens the importance of the molecular regulation of the enzyme in cardiovascular diseases (208, 209). NOX activity in nonphagocytic cells such as cardiovascular cells is acutely increased by diverse pathophysiological stimuli, including: (i) G protein-coupled receptor agonists, for example, angiotensin II and endothelin-1; (ii) cytokines, for example, TNF-α and TGF-β (transforming growth factor-β); (iii) growth factors, for example, thrombin, vascular endothelial growth factor (VEGF), and insulin; (iv) "metabolic" factors, for example, oxLDL, nonesterified (free) fatty acids, and glycated proteins; (v) hypoxia–reoxygenation or ischemia–reperfusion; and (vi) mechanical stimuli, for example, oscillatory shear (210).

The phagocytic NOX consists of a membrane-associated cytochrome b558 that comprises a large subunit, $gp91^{phox}$ ("phox" being derived from phagocytic oxidase), and a small subunit, $p22^{phox}$. Besides these, there are at least three cytosolic subunits ($p47^{phox}$, $p67^{phox}$, and $p40^{phox}$) and a low-molecular-weight G protein (Rac1 or Rac2) present. The subunits $p47^{phox}$, $p67^{phox}$, and $gp91^{phox}$ (NOX2) have been identified in vascular cells. However, several studies have confirmed that $p22^{phox}$ is present in all NOX systems and that this subunit is

essential for the functionality of the enzyme. Upon cell stimulation, $p47^{phox}$ becomes phosphorylated on multiple sites with several kinases (protein kinase C [PKC], protein kinase A, or MAPK) and the cytosolic subunits form a complex which migrates to the membrane, where it binds to the cytochrome b558. Then electrons are transferred from the substrate, NADPH, to $O_2$, leading to $O_2^{\bullet-}$ generation (211, 212). Phosphorylation and translocation of $p47^{phox}$ allows its interaction with $p22^{phox}$ and facilitates the binding of $p67^{phox}$ to cytochrome b558 (205). In addition, another key posttranslational modification involved in oxidase activation is Rac activation.

Several homologues of $gp91^{phox}$ have recently been reported to be expressed in nonphagocytic cells. Other members of the NOX family include NOX1, NOX2, NOX3, NOX4, and NOX5, as well as larger and more complex homologues termed DUOX1 and DUOX2 (206). NOX1 to NOX5 have 65-kDa core proteins, whereas DUOX1 and 2 have 175- to 180-kDa proteins that have a domain homologous to $gp91^{phox}$ as well as an additional peroxidase domain. Using this new terminology, NOX2 represents the neutrophil $gp91^{phox}$. The first homologue of $gp91^{phox}$, namely NOX1, was found to have significant proliferative activity and was also known by the alternative term mitogenic oxidase or MOX-1 (213).

Functionally endothelial NOX shares some but not all of the characteristics of neutrophil NOX. One major difference is that endothelial NOX continuously generates a low level of $O_2^{\bullet-}$, even in unstimulated cells, although its activity can be further increased by several agonists. However, neutrophil NOX primarily produces $O_2^{\bullet-}$ if the cells are stimulated. In regard to the isoform of NOXs, $gp91^{phox}$ (NOX2) has been considered as the major isoform of NOX proteins in vascular endothelial cells (214). The functional role of this NOX isoform has been confirmed by decrease in phorbol ester-induced $O_2^{\bullet-}$ production and endothelium-dependent relaxation in $gp91^{phox-/-}$ mice (215). In addition to $gp91^{phox}$, NOX4 mRNA is also detectable in endothelial cells. It appears that NOX4-dependent oxidase functionally contributes to the basal $O_2^{\bullet-}$ production in endothelial cells (216).

El Bekay et al. (217) have linked the increased generation of ROS, NADPH-oxidase activation, and the moderate increase of brain oxidative stress as key pathological components in the mouse model of fragile X syndrome. Although there are multiple ROS sources in the brain, NOX is one of the most important enzymes.

ECTO-NOX (because of their cell surface location) proteins comprise a family of NAD(P)H oxidases of plants and animals that exhibit both oxidative and protein disulfide isomerase (PDI)-like activities. They have no flavin, heme, nor nonheme iron prosthetic groups and do not require ancillary proteins for activation. ECTO-NOX proteins achieve protease (including proteinase K) resistance, contain a copper site, and form amyloids; all of these characteristics are also characteristics of prions (218). Molecular oxygen and protein disulfide both have been shown to function as electron acceptors for ECTO-NOX protein-catalyzed reactions. ECTO-NOX proteins are essential

to three very important areas of cell function which are to drive the enlargement phase of cell growth; as a terminal oxidase for plasma membrane electron transport, and as the biochemical core oscillator of the biological clock. An age-related ECTO-NOX protein (arNOX) found in human sera and buffy coat fractions of individuals >60 years generates superoxide as measured by reduction of ferricytochrome c and is capable of oxidizing circulating lipoproteins and other extracellular targets with a potential role in atherosclerosis (219).

It is well known that neutrophils are phagocytic cells involved in the nonspecific defense and inflammation. As mentioned, by appropriate stimuli, a significant production of superoxide anion, hydrogen peroxide, singlet oxygen, and hydroxyl radicals occurs. These ROS are produced from oxygen during the respiratory burst, and their formation involves both hexose monophosphate shunt and NOX activity (220, 221). PKC is essential for ligand-initiated assembly of neutrophil NOX for the generation of superoxide anion (222–224). Thus, the direct activation of PKC by a specific PKC activator, phorbol 12,13-dibutyrate, induced a remarkable $O_2^{\bullet -}$ generation, indicating that PKC may regulate entirely the $O_2^{\bullet -}$ synthesis. Martins et al. (225) studied the possible correlations between PKC activity, NOX phosphorylation, and ROS production in relation to age. Normal subjects ranging in age from 20 to 80 years were divided into six age groups in order to study the ROS production during PKC activity in granulocytes. VSMCs express two proteins, NOX1 and NOX4 (226), which are homologues to the gp91$^{phox}$ catalytic subunit in phagocytes (214). Biochemical differences include kinetics of activation, output, and regulation of NAD(P)H-dependent ROS production (227). In contrast to the activated phagocyte oxidase, which produces large quantities of ROS into an extracellular (phagosomal) compartment in inducible bursts, NOX1 and NOX4 generate low-level, predominantly intracellular ROS constitutively and in response to agonists (228). Although NOX1 and NOX4 generate ROS, overexpression of these two homologues in the same fibroblast suggests that NOX1 has mitogenic activity and is growth promoting (229), whereas NOX4 is implicated in cellular senescence (230). In VSMC, NOX1 is inducible and upregulated by growth factors and hormones, whereas NOX4 is downregulated by these molecules (231). The differential expression and growth factor-related responses of multiple NOX proteins in VSMC imply distinct mechanisms of NOX1 and NOX4 regulation. This might involve specific signaling pathways such as the biphasic production of angiotensin II-mediated ROS consisting of a rapid PKC-dependent phase, followed by a sustained Rac/Src/PI3 kinase-dependent phase, and may implicate more than one NOX enzyme in ROS generation in VSMC (232). Another potential mechanism for the differential regulation and function of the NOX proteins is the targeting of the isoforms to specific subcellular signaling domains. The two candidate sites for NAD(P)H oxidase localization are caveolae, flask-shaped plasma membrane invaginations which are enriched in cholesterol and an array of signaling molecules (233), and focal adhesions, which are major

sites of tyrosine kinase signaling that link the extracellular matrix (ECM) to cytoskeletal proteins through transmembrane integrins (234). Caveolae and focal adhesions are proposed sites for growth factor (233) and integrin (235) signaling, respectively.

Hilenski et al. tested the hypothesis that the opposing growth and senescence functions for NOX1 and NOX4 are caused by their differential subcellular locations (236). They found that NOX1 is colocalized with caveolin in caveolin-enriched fractions on the cell surface, NOX4 is colocalized with vinculin in focal adhesions and nuclei, and $p22^{phox}$ is found in patterns similar to NOX1 and NOX4. This targeting of NAD(P)H oxidase subunits to specific multimolecular signaling domains provides a possible explanation for their apparent differential roles in normal growth, differentiation, and disease.

***1.1.3.2 Lipoxygenases*** LOXs are a family of iron-containing enzymes that catalyze the dioxygenation of PUFAs in lipids containing a *cis*,*cis*-1,4-pentadiene structure, creating a family of biologically active lipids, such as prostaglandins (PGs), thromboxanes (TXs), and leukotrienes, which participate in inflammatory reactions and increase the permeability of vessels. In experimental models, 15-lipoxygenase was shown to induce LDL oxidation by enzymatic and nonenzymatic reactions. Experimental animals with an absence of the 15-lipoxygenase gene or reduced expression of 5-lipoxygenase are protected from lesions such as those found in animals with apolipoprotein E and LDL-receptor deficiency (237). Clinical data demonstrate that various genotypes of 5-lipoxygenase promoter are found in patients with atherosclerotic lesions or inflammation (238). 5-Lipoxygenase has been identified as an inducible source of ROS production in lymphocytes (239). The numbers in specific enzyme names such as 5-LOX, 12-LOX, or 15-LOX refer to the arachidonic acid site that is predominantly oxidized (240). 5-LOX is best known for its role in the biosynthesis of the leukotrienes A4, B4, C4, D4, and E4. The oxidized metabolites generated by 5-LOX were found to change the intracellular redox balance and to induce signal transduction pathways and gene expression.

LOX produce chiral LOOHs. All LOX require free PUFAs as substrates; only the 15-LOX can use bound fatty acids (240). Although the numbers of LOX indicate the carbon atom of arachidonic acid, LOX enzymes also use linoleic acid as a substrate. In case of a high LOOH production as a result of high free PUFA concentration, LOX might inactivate themselves. As a consequence, iron ions are liberated, which might start an LPO chain reaction by decomposing LOOH, generating a number of products including $LOO^{\bullet}$ radicals, epoxides, and α-, β-, γ-, or δ-unsaturated aldehydes, which in turn might modify proteins (134).

***1.1.3.3 Protein Kinases*** Protein kinase enzymes modify proteins by phosphorylation. Therefore, kinases are not leading directly to protein oxidation, but due to the triggered signaling events, might induce/activate enzymes,

producing oxidants. Phosphorylation usually results in a functional change of the target protein by changing enzyme activity, cellular location, or association with other proteins. Almost half of all human proteins may be modified by kinases, and kinases are known to regulate the majority of cellular pathways, especially those involved in signal transduction. Most kinases act on both serine and threonine, others act on tyrosine, and a number (dual-specificity kinases) act on all three (241).

Several MAPK pathways exist to facilitate transduction of biochemical signals across the plasma membrane and toward the nucleus (242). The p38 MAPK is the distal kinase in a major signal transduction pathway responsive to cytokines, growth factors, and environmental stress (e.g., exposure to oxidants or osmotic shock; (243, 244)). Activation of p38 is accomplished by dual phosphorylation of Thr 180 and Tyr 182 within a conserved domain (245). Once activated, p38 translocates to the nucleus, where it phosphorylates various transcription factors involved in the expression of proinflammatory gene products and execution of apoptotic programs (246). Numerous studies indicate that ROS are used as messenger or effector molecules to activate protein kinase modules, including the p38 cascade (247). For instance, p38, Jun N-terminal kinases (JNKs), and extracellular signal regulated kinase (ERK 1/2), are phosphorylated (activated) by exposure to exogenous $H_2O_2$ (248). Gonzalez et al. (249) determined whether age-associated changes in 5′ adenosine monophosphate-activated protein kinase (AMPK) might contribute to the poor stress tolerance of aged cardiac and skeletal muscle. The heart and gastrocnemius muscle were chosen for study so that comparisons between a highly oxidative muscle (the heart) and a highly glycolytic muscle (gastrocnemius) were made. In cultured human fibroblasts and yeast, the activity of AMPK or its yeast homologue, Snf1, was shown to increase with age (250, 251). Moreover, genetic mutations in the AMPK genes caused severe dysfunction of cardiac and skeletal muscles, suggesting that alterations in AMPK have clinical consequences and may potentially contribute to the decline in stress tolerance observed with aging.

Robinson et al. (252) showed that interleukin-1β (IL-1β), $H_2O_2$, and sorbitol-induced hyperosmolarity mediate a 5- to 10-fold increase in phosphorylation (activation) of the p38 protein kinase in rat primary glial cells as measured by analyses of Western blots using an antibody directed against the dually phosphorylated (active) p38.

***1.1.3.4 Mixed-Function Oxidases*** The production of some oxidants is catalyzed by one of several different enzyme systems that are variously referred to as mixed-function oxidation (MFO) systems (253) or metal-catalyzed oxidation (MCO) systems (254). These systems include a large class of flavoproteins, reduced forms of [NAD(P)H] oxidases, the reduced form of nicotinamide adenine dinucleotide quinone reductase, dehydrogenases, and CYP450 reductases, which, in intermediary metabolism, normally serve as electron carriers between various metabolic reactions. However,

during oxidative stress or if an appropriate electron acceptor is missing, the reduced forms of the abovementioned enzymes are able to react with $O_2$ to form ROS (29). Often in the following redox cycle, $Fe^{2+}$ is involved and highly reactive species are formed ($^{\bullet}OH$) that are able to oxidize amino acids directly at the metal-binding site of the enzyme, therefore inhibiting the enzymatic activity and often rendering the inactivated enzyme to preferential degradation (255).

Therefore, virus NAD(P)H oxidases and reductases, including CYP450s, might lead to the oxidation of numerous proteins. In addition to that, xanthine oxidase, horseradish peroxidase, and glucose oxidase are also able to do so (256). GS of *E. coli* was demonstrated to be inactivated by MCO systems, including nonenzymatic systems comprised of either ascorbate, $O_2$ and $Fe^{3+}$, or $Fe^{2+}$ and $O_2$; and enzymatic systems such as rabbit liver microsomal CYP450 reductase together with CYP450 isozyme 2 [P-450(LM2)], microbial NADH oxidase, putidaredoxin reductase together with putidaredoxin with or without CYP450, xanthine oxidase together with ferredoxin or putidaredoxin, and partially purified enzymes (NADH oxidase) from *Klebsiella aerogenes* or *E. coli*. Inactivation of GS by all enzyme systems was shown to be dependent on $O_2$ and NAD(P)H (except in the case of xanthine oxidase, for which hypoxanthine serves as an electron donor). All systems are stimulated by $Fe^{3+}$ and inhibited by catalase, Mn(II), EDTA, o-phenanthroline, and histidine (257). Inactivation of GS by either the ascorbate system or the NADH oxidase system is associated with the modification of a single histidine residue in each GS subunit.

Fucci et al. (253) found that 10 enzymes (alcohol dehydrogenase, Aspartokinase III, creatine kinase, enolase, GS, glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, phosphoglycerate kinase (PGK), and pyruvate kinase) were inactivated by both MFO systems NADH oxidase and CYP450. All of the inactivation reactions required NAD(P)H and were inhibited by catalase. It is noteworthy that most of the susceptible enzymes are either synthetases, kinases, or NAD(P)-dependent dehydrogenases; that is, they possess a nucleotide-binding site at the catalytic center. In addition, they require divalent metal cations for activity and contain a histidine residue at or near the catalytic site. The inactivation of enzymes by MFO systems could occur by the mechanism demonstrated in Figure 1.17 (reactions 39–42).

A central role of hydrogen peroxide was often demonstrated by the blocking effect of catalase. The involvement of iron was demonstrated by the inhibiting action of chelators, whereas a role of $Fe^{2+}$ is underlined by the fact that $Fe^{2+}$ and $O_2$ also inactivates the GS (257). In contrast, $Fe^{3+}$ has no such effect regardless of the presence of oxygen. The fact that the inactivation of GS is associated with the loss of just 1 of 16 histidine residues in each subunit is a clear indication of a site-specific event. In addition to that, also the inactivation of other enzymes, as PGK or mammalian SOD, is due to modification of a single histidine. A site-specific binding of $Fe^{2+}$ that attacks a histidine in the catalytic center or nearby was suggested (253).

| | |
|---|---|
| $NAD(P)H + H^+ + O_2 \xrightarrow{MFO} H_2O_2 + NAD(P)^+$ | **Reaction 39** |
| $NAD(P)H + 2\ Fe^{3+} \xrightarrow{MFO} 2\ Fe^{2+} + NAD(P)^+ + H^+$ | **Reaction 40** |
| $Fe^{2+} + H_2O_2 \rightarrow$ activated oxygen $+ Fe^{3+}$ | **Reaction 41** |
| Activated oxygen + enzyme $\rightarrow$ oxidized enzyme | **Reaction 42** |

**FIG. 1.17** The inactivation of NAD(P)-dependent dehydrogenases, possessing a nucleotide binding site at the catalytic center and requiring both divalent metal cations and a histidine residue at or near the catalytic site for their functionality, may be inactivated by mixed-function oxidation (MFO) systems that could work according to the depicted mechanism (**reaction 39–42**) (according to Stadtman, Arch. Biochem. Biophys. 423: 2–5, 2004).

Stadtman and coworkers studied the role of protein modification in a large number of systems in the aging process, using both enzymatic and nonenzymatic MFO systems as protein-modifying agents (258). MFO caused in many enzymes a decrease in activity and enhanced proteolytic susceptibility (259). Enzymes readily oxidizable by MFO are also found to be oxidized in the physiological aging process. This led Oliver et al. (31) and Stadtman (255) to the suggestion of a leading role of MFO in age-related protein oxidation. Zhou and Gafni (260) tested whether reduction after MFO may result in a protein identical to the native enzyme. PGK was exposed to an ascorbate : $FeCl_3$ system, which leads to an inactivation of the enzyme. This can be partially reversed by addition of 2-mercaptoethanol. It was proposed that the selective oxidation of the reactive Cys residues in PGK was having a role in PGK inactivation. This was confirmed by the fact that blocking of these cysteines by methylation was shown to protect PGK (261).

It was summarized in Reference 262 that a variety of MFO systems might catalyze the oxidative inactivation of enzymes mediated by site-directed Fenton chemistry.

Mattana et al. (263) demonstrated that mesangial matrix proteins can undergo oxidative modification *in vitro* using an MFO system, as evidenced by increased carbonyl content. Adhesion of macrophages to mesangial matrix appears to be enhanced by oxidation of this substrate, an effect that may be mediated via interaction of macrophage scavenger receptors with oxidized matrix proteins. Oxidative modification of mesangial matrix may play a role in the pathogenesis of glomerulosclerosis by promoting accumulation of macrophages in the mesangium.

***1.1.3.5 Nitric Oxide Synthetase (NOS)*** NOS enzymes act as a catalyst to convert L-arginine to nitric oxide and L-citrulline. There are three recognized

isoforms of this enzyme, two of which are constitutive forms: neuronal NOS (nNOS, NOS1) (264) and endothelium NOS (eNOS, NOS3) (265); the third one is the inducible form (Inducible nitric oxide synthase [iNOS], NOS2) (266). Many tissues express one or more of these isoforms. Each isoform varies in its tissue specificity; several isoforms can be found in the same tissue but might have different functions. Therefore, eNOS can be stimulated by shear stress in the vascular endothelium (267), while iNOS may occur in normal epithelium. The constitutive NOS activity are controlled by the intracellular calcium concentration, whereas the iNOS is independent of the cytosolic calcium concentration due to the permanent binding of calmodulin (CaM). The other forms are binding CaM permanently (268). The isoform iNOS is inducibly expressed in macrophages after stimulation by cytokines, lipopolysaccharides (LPSs), and other immunologically relevant agents (269). Expression of iNOS is regulated at the transcriptional and posttranscriptional level by signaling pathways that involve agents such as the redox-responsive transcription factor NFκB or MAPKs (270).

The recent discovery of •NO production by a mitochondrial NOS (mtNOS) added a new isoform of the enzyme and changed the view on both regulation of tissue oxygen uptake and of free-radical toxicity. It has been reported that this NOS isoform is expressed in a constitutive manner and is located in the inner membrane of rat liver mitochondria (100).

NOS enzymes are the major sources of •NO production. In 1977, Arnold et al. (271) demonstrated the ability to dilate blood vessels and relax smooth muscle tissue. In 1992, •NO was the "molecule of the year" (272). Six years later, the Nobel Prize in Physiology and Medicine (273) was given for the identification of •NO as a signaling molecule. In the succeeding years, the role of •NO in signaling cascades, vasodilation, and immune response was demonstrated. •NO is an uncharged lipophilic molecule (268). Its reactivity is limited, but it undergoes reactions with oxygen, GSH, and superoxide radicals. Nitric oxide can act as an electron donor (oxidant) or an electron acceptor (antioxidant). •NO is able to diffuse across cellular membranes and cell layers. The guanylate cyclase binds nitric oxide and produces cGMP upon this activation. cGMP regulates the activity of a number of target proteins (274), including cGMP-dependent protein kinase (275), cGMP-regulated phosphodiesterase (276), and cGMP-gated ion channels (277).

nNOS plays a direct role in the physiological activity of skeletal muscle contraction. While nNOS and eNOS may be activated during repetitive muscle contractions (278), nNOS is probably the predominant •NO producer during contractile activity (279). eNOS is located primarily in endothelial tissue, cardiocytes, and some groups of neurons (268). eNOS is associated with skeletal muscle mitochondria (278). •NO production from eNOS regulates a number of physiological processes, including platelet aggregation or vasorelaxation (268).

iNOS is located in macrophages and is induced by endotoxins or inflammatory cytokines (274). Therefore, high levels of •NO are released by iNOS in

response to inflammatory stimuli. Such a high •NO production produced by iNOS has been proposed to contribute to certain disease pathways. Therefore, in stroke, an increased activity of iNOS was demonstrated, although it is well known that high levels of nitric oxide and peroxynitrite are neurotoxic (280). The radical scavenger α-phenyl-N-tert-butyl nitrone (PBN) inhibits •NO production by interfering with the *de novo* iNOS synthesis after cytokine stimulation (281).

Renal aging is associated with a progressive decline of kidney function and increased vascular resistance. Interestingly, the kidney has high levels of iNOS in epithelial and vascular cells. As a result, De Lutiis et al. (282) have investigated mRNA and protein iNOS expression and localization and nitric oxide (•NO) production in young and aged rats. An increased expression of iNOS mRNA and protein were shown to occur in rat kidney during aging. In the aged rat kidney, the production of •NO decreased due to the reduction of the activities of the three NOS. This suggests that in the aged rat, a progressive increase of superoxide anion does not imply an increase in the production of •NO, which functions as a scavenger molecule, causing oxidative stress with accumulation of ROS.

One regulatory mechanism for NOS is substrate (L-arginine) bioavailability. Steppan et al. (283) tested the hypothesis that arginase (Arg), which metabolizes L-arginine, constrains NOS activity in the cardiac myocyte in an isoform-specific manner. They concluded that mitochondrial Arg II negatively regulates NOS1 activity, most likely by limiting substrate availability in its microdomain. These findings have implications for therapy in pathophysiologic states such as aging and heart failure, in which myocardial •NO signaling is disrupted.

In the sarcoplasmic reticulum (SR), NOS1 colocalizes with the ryanodine receptor (RYR), and activation of NOS1 positively modulates cardiac contractility. Moreover, NOS1 deficiency leads to an increase in xanthine oxidase-dependent ROS activity, which dramatically depresses myocardial contractile function (284). In contrast, the NOS3 isoform coupled to the $\beta_3$-adrenergic receptor (AR) inhibits L-type $Ca^{2+}$ channels, and thus inhibits β-AR-mediated increases in myocardial contractility (285).

•NO signaling may be mediated by a soluble guanylyl cyclase-dependent increase in cGMP (286) or cGMP-independent nitrosylation of a broad spectrum of effector proteins (287). An emerging body of evidence indicates that the balance between •NO and $O_2^{\bullet -}$ regulates the •NO/redox balance, thus determining the nitrosylation of proteins and their resultant physiological or pathophysiological effects (288).

An emerging paradigm in •NO biology indicates that Arg, an enzyme that also uses L-arginine as a substrate, reciprocally regulates NOS activity. This phenomenon has been demonstrated for both constitutive (289) and inducible (290) NOS isoforms, where Arg constrains (and, thus, regulates) NOS activity. Also, upregulation of Arg has been shown to contribute to the pathophysiology of disease processes, in which •NO signaling is dysregulated (e.g., the endothelial dysfunction of aging (289), hypertension (291), atherosclerosis

(292), the erectile dysfunction in diabetes (293), and reactive airways disease in asthma (294)).

It is established that •NO modulates the activity of a number of key ion channels and proteins that regulate $Ca^{2+}$ release and thus modulate excitation–contraction coupling. Moreover, •NO can either accentuate or attenuate myocardial contractility. This complex and sometimes directionally opposite effect of •NO is accomplished by different NOS isoforms being localized to specific cellular microdomains. In this regard, NOS1, localized to the SR (284), is associated with the RYR and sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) receptors, where it augments $Ca^{2+}$ release in response to frequency (295) and β-AR stimulation (296). In contrast, NOS3 localized to sarcolemmal caveolae (297) negatively regulates L-type $Ca^{2+}$ channels and attenuates the response to $\beta_1$-AR activation (298). This effect is mediated by $\beta_3$-ARs, which are coupled to the NOS3 isoform (299). These dual and opposing effects of $\beta_3$-AR activation are cGMP/guanylyl cyclase-dependent. In contrast, the mechanisms underlying the effects of NOS1/•NO on SR $Ca^{2+}$-release are mediated by alterations in nitrosylation of the RYR and possibly the SERCA channels (300).

iNOS is regulated at the transcriptional level, and its gene promoter has binding sites for multiple transcription factors, including NFκB (301). mtNOS is a fine regulator of oxygen uptake and ROS that eventually modulates the activity of regulatory proteins and cell cycle progression. Elfering et al. (302) reported 100% homology between liver mtNOS and nNOS by mass spectrometry; differentially, liver mtNOS has two posttranslational modifications: acylation with myristic acid and phosphorylation at C-terminus, and a lower molecular weight (130 vs. 157 kDa). The results from Carreras et al. (303) suggest that a synchronized increase of mtNOS and derived $H_2O_2$ operate on hepatocyte signaling pathways to support the liver developmental transition from proliferation to quiescence.

An excessive production of •NO by the iNOS due to pro-inflammatory responses has been considered a feature of neurodegenerative disorders and brain aging (304). In contrast, a decreased production of •NO by the mtNOS has been recently proposed as the cause of decreased mitochondrial biogenesis and turnover with direct implication in brain aging (305–307).

The mtNOS activity was simultaneously reported in rat liver mitochondria by Giulivi et al. (308) and by Ghafourifar and Richter (309). Crossed immunoprecipitation and kinetic evidence suggest that, according to the supercomplexes model of the mitochondrial respiratory chain, mtNOS is structurally attached to both complex I (NADH-ubiquinone reductase) and to complex IV (cytochrome oxidase) (310).

***1.1.3.6 Myeloperoxidase*** MPO, a heme protein existing in neutrophils and monocytes, is implicated in various stages of inflammatory conditions with the production of a variety of potent oxidants. MPO is a key enzyme of neutrophils to produce potent oxidants whose uncontrolled formation leads to protein oxidation, including hypochlorous acid (HOCl), tyrosyl radical (Tyr•),

and nitrogen dioxide ($NO_2^{\bullet}$), acting as a bactericidal system against invading organisms (311, 312).

Many lines of evidence have implicated the role of MPO in promoting oxidative stress in many inflammatory diseases, including ischemia–reperfusion injury, atherosclerosis, rheumatoid arthritis, periodontal disease, and proteinuric glomerulopathies (313–315). In addition, MPO generates a family of tyrosyl radical-added products, and afterward, dityrosine might be formed through interaction with two tyrosyl radicals (316, 317). Proteins and lipids are damaged by tyrosyl radicals from MPO-derived tyrosine oxidation, that is, tyrosylation might play a role in the pathogenesis of many diseases (318). Many studies reported that protein oxidation via the MPO/$H_2O_2$ system and hypochlorite are major oxidants generated by neutrophils and macrophages activated at inflammatory sites, such as in atherosclerotic lesions.

Son et al. (319) investigated the effect of aging on MPO in the kidney and documented that both activity and protein level of MPO and dityrosine formation were increased in *ad libitum*-fed rats during aging. Dityrosine, one of the specific biomarkers for MPO activity, is formed via tyrosyl radical intermediates which can be generated by MPO (317). Increased MPO activity with aging may be related to increased recruitment of inflammatory cells, contributing to higher levels of protein oxidation in the aging process.

Another major endogenous oxidizing species is MPO-derived HOCl. Interaction of this molecule with Tyr, Trp, Lys, and Met residues leads to formation of chlorotyrosine, chloramines, aldehydes, and Met-sulfoxide (320).

***1.1.3.7 Cyclooxygenase*** Cyclooxygenases (COXs) catalyze the rate-limiting reaction to produce PGs, prostacyclin, and TX. These enzymes are members of a heme enzyme family and they also possess peroxidase activity (321). Two different isoforms of COX, COX-1 and COX-2, catalyze the conversion of arachidonic acid to prostaglandins $PGE_2$, $PGI_2$, $PGF_2$, $PGD_2$, and $TXA_2$ (322), which are potent activators of a large family of G protein-coupled receptors and mediate specific biological responses in various tissues and cells (323).

COX-1 is the widely expressed constitutive form, and COX-2 is the inducible form that is upregulated by cytokines and mitogens. Classically, COX-1 has been considered as the constitutively expressed isoform, primarily responsible for homeostatic PG synthesis, and COX-2 as the isoform induced in response to inflammatory stimuli, and thus the most appropriate target for anti-inflammatory drugs (324, 325). COX-1 has been implicated in ROS production in cells stimulated with TNF-α, IL-1, bacterial LPS, or the tumor promoter 4-O-tetradecanoylphorbol-13-acetate (TPA) (326). A significant part of LPS-induced neurotoxic processes is mediated by oxidative damage, which can be evaluated by assessing protein carbonyls and nitrotyrosine levels. Choi et al. (327) investigated whether protein carbonyls and nitrotyrosine levels were altered in COX-$1^{-/-}$ mice 24 h after LPS injection. Levels of protein carbonyls were significantly increased in LPS-injected compared with

vehicle-injected COX-$1^{+/+}$ mice. However, in LPS-injected COX-$1^{-/-}$ mice, there was no significant change in protein carbonyls compared with vehicle-injected COX-$1^{-/-}$ mice. Nitrotyrosine immunoreactivity was increased in the hippocampus of LPS-injected COX-$1^{+/+}$ mice compared with vehicle-injected COX-$1^{+/+}$ mice. In contrast, very few nitrotyrosine immunoreactive cells were detected in the hippocampus of LPS-injected COX-$1^{-/-}$ mice.

COX-2 expression is linked to the progression and severity of pathology in AD. Epidemiological studies indicate that nonsteroidal anti-inflammatory drugs (NSAIDs), inhibitors of COX, delay the onset of AD (328). Ibuprofen, a nonselective COX inhibitor, attenuates plaque pathology in Tg2576 mice (329). Neuronal COX-2 expression is increased in the affected regions of AD brain (330), correlating to the severity of AD pathology (331). COX-2 overexpression in primary neurons potentiates β-amyloid (Aβ) neurotoxicity *in vitro* (332). Therefore, COX-2 may interact with the metabolism of Aβ in AD. COX harnesses two enzymatic activities to produce prostaglandin H2 (PGH2). Authentic "cyclooxygenase" activity first converts arachidonic acid to prostaglandin G2 (PGG2), and subsequent peroxidase activity reduces PGG2 to PGH2. The peroxidase reaction of COX is analogous to that of horseradish peroxidase and can utilize a wide range of hydroperoxides, including $H_2O_2$, rather than PGG2, as substrates. The cycle of the peroxidase reaction can occur independently of COX activity, utilizing hydroperoxides such as hydrogen peroxide (321). During the peroxidase reaction, ferric heme of the resting enzyme is oxidized to form ferryl iron and a porphyrin radical. The porphyrin radical is reduced to form a tyrosyl radical at $Tyr^{385}$ by an intramolecular electron transfer.

Nagano et al. (333) hypothesized that the sole tyrosine residue of human Aβ could be a target for the peroxidative activity of COX-2 and that tyrosyl radicals of human Aβ formed by the reduction of oxidized COX-2 will cross-link each other to generate dityrosine (83), and they investigated the possibility of direct interaction between human Aβ and COX-2 being mediated by the peroxidase activity. Human Aβ formed dimers when it was reacted with COX-2 and hydrogen peroxide. Moreover, the peptide formed a cross-linked complex directly with COX-2. They examined the effect of COX-2 on Aβ oligomerization. Their data indicate that the peroxidative activity of COX-2 induces the dimerization of human Aβ by a $H_2O_2$-mediated mechanism, and the enzyme itself also cross-links with human Aβ directly. NSAIDs such as indomethacin and flurbiprofen specifically suppress the radical formation at $Tyr^{385}$ of COX, suggesting that NSAIDs inhibited COX-2-Aβ cross-linking by decreasing tyrosyl radicals in COX-2 in this study.

### 1.1.4 Protein Oxidation in Cells and Cellular Structures

***1.1.4.1 Protein Oxidation in Blood and Blood Cells*** Circulating blood and blood cells may be major targets of protein oxidation, and it was shown that the levels of oxidatively modified proteins increase with age in circulating

erythrocytes. Erythrocytes fractionated by their age, for example by cell density, demonstrated an enhanced amount of protein carbonyls in higher densities (31). In general, senescent red blood cells (RBCs) are removed from the blood circulation by the reticuloendothelial system in the liver and spleen (334, 335). Several studies have demonstrated the involvement of binding of antiband 3 IgG autoantibody to band 3 glycoprotein on the surface of senescent erythrocytes (336, 337). Macrophage phagocytosis of normal RBCs is negatively regulated by CD47. RBC senescence is associated with several physical and chemical alterations such as an increased density, loss of lipid asymmetry, accumulation of LPO products in the plasma membrane, formation of senescent cell antigens (SCAs), and an increased amount of immunoglobulins and complement factor C3b on the cell surface (338). *In vitro* oxidation of RBCs results in aldehyde formation and membrane protein cross-linking, LPO, and possibly also conjugation of lipid fragments to proteins and protein degradation (339, 340). Sialosaccharide chains of glycophorin, which were found to cluster or aggregate in the membrane of senescent RBCs, represent one candidate ligand for recognition of oxidized or aged RBCs by macrophage scavenger receptors (341). Olsson and Oldenborg (342) found that phagocytosis of oxidatively damaged RBCs (ox-RBCs) were critically dependent on serum. Indeed, serum proteins such as complement or IgG were shown to be involved in the uptake of senescent RBCs (343, 344). The data from the study of Olsson and Oldenborg (342) indicated a high capacity of CD47 to form clusters on the RBC surface following antibody cross-linking. However, clustering of CD47 was much less evident on ox-RBCs, possibly due to oxidation-mediated aldehyde formation and membrane protein cross-linking. Anemia might be caused by increased vulnerability of erythrocytes due to a SOD1 deficiency. Since mammalian erythrocytes lack nuclei, they are incapable of replacing damaged proteins. In addition, while most mammalian cells possess two intracellular SOD isoforms to protect against ROS (345), erythrocytes lack mitochondria and, as a result, carry only the SOD1 protein to scavenge superoxide anions. An SOD1 deficiency leads to an increased erythrocyte vulnerability by the oxidative modification of proteins and lipids, resulting in anemia and compensatory activation of erythropoiesis.

The continuous destruction of oxidized erythrocytes appears to induce the formation of autoantibodies against certain erythrocyte components, for example, carbonic anhydrase II, and the immune complex is deposited in the glomeruli (346). A deficiency in other antioxidative proteins, such as peroxiredoxins (Prx) PrxI (347) and PrxII (348), which catalytically function as Trx-dependent peroxidases, also causes anemia by affecting the life span of erythrocytes. The data from Iuchi et al. (346) showed that the erythrocyte is the preferred target of oxidative modification in the blood and is prone to degradation, leading to anemia. Since oxidized erythrocyte components are antigenic in regards to the formation of autoantibodies, a long-term exposure to severe oxidative stress consequently causes an autoimmune response to oxidized erythrocytes that can be regarded as an acquired antigen by oxidative

modification. The continuous oxidation and destruction of erythrocytes would ultimately produce a sufficient amount of antibodies to generate autoimmune diseases. Seppi et al. (349) investigated the occurrence of membrane protein oxidation during senescence of human erythrocytes (350). The thiol state of proteins was also evaluated because it is known that activity of many integral proteins like ion transporters is affected by sulfhydryl group oxidation (351). Glycation is among the major causes of heterogeneity in human hemoglobin. In hemoglobin, glucose reacts predominantly with the aminoterminal valine of the β chains, to a lesser extent with the aminoterminal valine of the α chains, and with several ε-amino lysines. Levels of glycated hemoglobin are a measure of long-term, time averaged plasma glucose concentration (352).

It has been suggested that RBC aging is affected under different blood bank conditions. The results of Antonelou et al. have provided circumstantial evidence for a lower protein oxidative damage in citrate–phosphate–dextrose–saline–adenine–glucose–mannitol stored RBCs compared to the citrate–phosphate–dextrose–adenine stored cells (353).

Besides erythrocytes, other cells are also affected during aging. Most of the HSPs have been found to be downregulated in lymphocytes from old donors in comparison to young subjects (354). The induction of HSP72 in response to heat shock was also decreased during aging in human lymphocytes and monocytes (355). Simar et al. (356) mainly showed that HSP72 is largely expressed in leukocytes of active, healthy, elderly people who maintained regular physical activity. However, as shown by the lower percentage of lymphocytes expressing HSP72 and the lower intracellular HSP72 expression in monocytes and granulocytes from the oldest people, the maintenance of physical activity in elderly subjects was not sufficient enough to reach the levels of HSP72 expression measured in active young subjects. In response to exercise, an increased percentage of HSP72-positive lymphocytes in the oldest people was observed. It was also interesting that older people also showed significant lower counts of lymphocytes than younger subjects (357).

Lymphocytes are the carriers of immunological specificity and, therefore, play an important role in the defense against environmental pathogens. A sophisticated combination of regulatory mechanisms ensures that even minute amounts of pathogen activate highly aggressive responses without causing major damage to the host tissue. The immune response typically involves the lymphocyte receptor for antigen, receptors for costimulatory signals, and various types of cytokines (358). The response is also subject to regulation by redox processes. The functional activation of T lymphocytes is strongly enhanced by ROS and/or by a shift in the intracellular GSH redox state (359). Superoxide and/or physiologically relevant concentrations of hydrogen peroxide were shown to augment the production of IL-2 by antigenically or mitogenically stimulated T cells in various experimental systems (360). Low micromolar concentrations of hydrogen peroxide were also shown to induce the expression of the IL-2 receptor in a mouse T-cell lymphoma line. In T cells, strong activation of the costimulatory receptor CD28 causes a significant

decrease in intracellular GSH levels and the endogenous production of hydrogen peroxide (239).

***1.1.4.2 Protein Oxidation of Glycolytic Enzymes and Mitochondria*** Mitochondria were brought to attention in aging biology due to their central role in producing chemical energy (adenosine triphosphate, ATP) for cellular requirements and the declines of basal metabolic rate and physiological performances, which are characteristic of aged mammals. Mitochondrial oxidative phosphorylation is a process that encompasses electron transfer between the complexes of the respiratory chain, vectorial $H^+$ release into the intermembrane space, and $H^+$ reentry to the matrix through $F_0$ with ATP synthesis by F1-ATP synthase. An age-dependent impairment of mitochondrial function may be due to either decreased electron transfer, increased $H^+$ permeability of the inner membrane, or decreased $H^+$-driven ATP synthesis (307).

The mitochondrial electron transport chain is the predominant site of ATP production and also the primary producer and target of ROS such as $O_2^{\bullet-}$ and $H_2O_2$. This notion is supported by the findings that the rates of mitochondrial $O_2^{\bullet-}/H_2O_2$ generation and amounts of oxidative damage to macromolecules are elevated during aging in different species (361, 362). A major site for the univalent reduction of molecular oxygen to superoxide is ubisemiquinone (363).

A common observation in mammalian aging studies is the decreased electron transfer in mitochondria isolated from old animals (305). Mitochondria isolated from the brain, liver, heart, and kidney of old rats and mice show decreased electron transfer activity in complexes I and IV, whereas complexes II and III are largely unaffected.

Oxidized and nitrated mitochondrial proteins are starting to be considered as inhibitors of mitochondrial biogenesis and as promoters of mitochondrial death and elimination through phagolysosomal recognition and internalization. Mitochondrial protein carbonyls were shown to increase in mitochondria from brain and other organs of aged animals (305, 306).

Mitochondria exposed to exogenous radicals lose control of ion balance, notably of calcium transport; it was proposed that protein oxidation as well as proteolysis may be important in such changes ((364, 365); see also http://www.ncbi.nlm.nih.gov/pubmed?term=%22Reinheckel%20T%22%5BAuthor%5D). Leakage of electrons from the transport chains, leading to radical fluxes and self-inactivation, may also be important, especially as mitochondria seem to be a major radical-generating site and contain more oxidized DNA than nuclei (366).

The hypothesis on the involvement of mitochondria in aging has some basis, such as (i) mitochondria are among the most important subcellular sites of oxyradical production, (ii) $O_2^{\bullet-}$-steady-state concentration in the mitochondrial matrix is about 5–10 times higher than in the cytosolic and nuclear spaces, (iii) mtDNA is in close proximity to the sites of oxyradical generation and is not protected by histones, and (iv) the accumulation of faulty synthesized

proteins and protein complexes might compromise energy transduction. Mitochondrial aging by oxyradical-induced damage would occur through mtDNA damage and relatively specific protein inactivation, such as adenine nucleotide translocase. This process would lead to a state of dysfunctional mitochondria that are not able to maintain membrane potential and ATP synthesis. Dysfunctional mitochondria release $Ca^{2+}$ and cytochrome c to the cytosol and signal for organelle ingestion by primary lysosomes and apoptosis (100).

It is known that oxidative phosphorylation is regulated in mitochondria by the availability of ADP, $O_2$, and $^{\bullet}NO$ in the matrix and at the N-side of the mitochondrial inner membrane (367). The modifications of mitochondrial proteins by oxidation, with formation of protein carbonyls, or by $^{\bullet}NO$-mediated reactions (S-nitrosylation and nitration) lead to a mitochondrial dysfunction associated with decreased electron transfer, decreased inner mitochondrial membrane potential, and decreased selective permeability with eventual induction of mitochondrial permeability transition (305, 306).

In addition to the mitochondrial electron transfer chain, the citric acid cycle plays a pivotal role in mitochondrial bioenergetics by providing the reducing equivalents—NADH and $FADH_2$ for ATP synthesis, and NADPH for the reduction of $H_2O_2$ and GSH disulfides in mitochondria—as well as supplying intermediates essential for fatty acid and protein synthesis. Because of its biochemical design, a decrease in the activity of a single enzyme can potentially diminish the turnover rates of the entire citric acid cycle or divert intermediates to other pathways. Metabolism through the citric acid cycle results in the net synthesis of intermediates (anaplerosis) as well as the export of intermediates from the mitochondria (cataplerosis) used in fatty acid and protein synthesis and potentially as extramitochondrial signal molecules (368).

In the study of Yarian et al. (368), a systematic approach was undertaken to identify protein targets of the aging process in mitochondria by determining losses in enzymatic activity of a central metabolic pathway, the citric acid cycle. Results of this study indicate that among citric acid cycle enzymes, aconitase exhibits the most significant age-associated decline in activity. A smaller, but significant decline occurred for α-ketoglutarate dehydrogenase while $NADP^+$-isocitrate dehydrogenase ($NADP^+$-ICD) showed an elevation in activity.

Experimental studies have shown that specific enzymes of the citric acid cycle are susceptible to oxidation. For instance, aconitase and α-ketoglutarate dehydrogenase can undergo oxidative modification *in vitro* with a subsequent decrease in enzymatic activity, following exposure of mammalian mitochondria to hydrogen peroxide (369–371). Aconitase has also been shown to be carbonylated *in vivo* in insects and mammals, resulting in a reduction in activity (34, 372, 373). $NADP^+$-ICD is a target of glycation, and subsequent inactivation, in kidney mitochondria from rats during the normal aging process and in a diabetes-induced rat model (374).

Recent reports identifying citric acid cycle intermediates as ligands for G-protein-coupled receptors suggest a link between the citric acid cycle and certain metabolic situations accompanying aging. Succinate has been shown

to be a natural ligand for an orphan receptor highly expressed in kidneys with a prohypertensive effect involving the renin–angiotensin system, while α-ketoglutarate is a ligand for a homologous receptor (375). It is of interest to point out that aging animals suffer from metabolic diseases such as hypertension, atherosclerosis, and diabetes, which may, in part, be affected by an age-associated alteration of the citric acid cycle and, consequently, intermediates acting as signaling molecules. Moreau et al. (376) measured the HNE adducts of the α-ketoglutarate dehydrogenase complex (KGDC) and determined that hearts from old rats exhibit significantly higher HNE modification. Xin et al. (377) used senescent (passage 45) and young (passage 3) pulmonary artery endothelial cells (PAECs). The data demonstrated that deficiency of complex IV in senescent cells enhanced oxidative and nitrosative stress, which may be responsible for senescence-induced endothelial cell loss and dysfunction. Complex IV has been found to be deficient in aged muscle, neurons, and endothelial cells *in vitro* and *in vivo* (378–380). For example, in cultures of senescent PAECs, gene expression and catalytic activity of complex IV were dramatically downregulated (381). Excessive $O_2^{\bullet -}$ can react with nitric oxide ($^{\bullet}NO$), endogenously generated in endothelial cells, to form peroxynitrite. Peroxynitrite can nitrate tyrosine or nitrosylate cystine residues of varieties of proteins in endothelial cells.

It is well accepted, that damage to mitochondrial DNA is an important contributor to human aging and cancer (382). Chemistry-based approaches in targeting mitochondria can be used to influence mitochondrial biochemistry and the biology of aging (383). Mitochondria has been previously identified to exhibit considerable age-associated increase in the level of protein carbonyls (384). Agarwal and Sohal (385) demonstrated an age-related increase in mitochondrial protein oxidation in flies.

ROS may be generated both within mitochondria and extramitochondrially. It is generally thought that mitochondrial dysfunction is a major player in aging and age-related tissue pathologies. Intramitochondrial damage may result from the direct effects of ROS on mitochondrial proteins or derive from their mutagenic effects on organelle DNA. In either case, altered protein species may be generated. Modification to the enzyme aconitase appears to be the best characterized mitochondrial protein, which undergoes oxidative damage during aging (373) and in exercised muscle (386, 387). Possible consequences of mitochondrial damage are either apoptosis or the age-related accumulation of cells possessing dysfunctional mitochondria.

*1.1.4.2.1 Glycolytic Enzymes* In the glycolytic pathway, glucose is converted into pyruvate. The free energy released in this process is used to form the high-energy compounds ATP and NADH (reduced nicotinamide adenine dinucleotide), and in the mitochondria, aerobic glycolysis takes place. Several enzymes play a role in this pathway and may be affected during aging. Gafni and Noy (388) compared the properties of rat muscle GAPDH from young and old rats. They found significant modifications of GAPDH in aged animals.

*1.1.4.2.2 Aconitase* Aconitase (aconitate hydratase) catalyzes the stereospecific isomerization of citrate to isocitrate via *cis*aconitate in the tricarboxylic acid (TCA) cycle, a nonredox active process (389). Some of the potential consequenses of loss of aconitase activity can be the slowing down of glycolysis and the TCA cycle, with consequent decrease in flow of electrons to oxygen, leading to depression of oxidative phosphorylation. Since the TCA cycle reduces $NAD^+$ to NADH at several points, a pro-oxidizing shift may occur in $NAD^+$/NADH ratio. Another consequence of the loss of aconitase activity would be the accumulation of citrate, which has been documented in insects and rats. Citrate can bind $Fe^{2+}$, which, in turn, can cause scission of $H_2O_2$ to generate the highly reactive hydroxyl free radical (390). Aconitase activity declined as a result of protein carbonyl formation during aging (34). Exposure of aconitase to superoxide or hydrogen peroxide causes the release of iron from the $[4Fe\text{-}4S]^{2+}$ cluster, inactivating the enzyme (391–393). In addition, $O_2^{\bullet-}$ and $H_2O_2$ do not appear to directly inhibit aconitase, instead requiring interaction between aconitase and a membrane component responsive to peroxide (370). The posttranslational oxidative modification, carbonylation, has previously been shown to result in a decrease in aconitase activity in both the housefly (34, 394, 395) and fruit fly, *Drosophila melanogaster* (372). In the house fly, aconitase was estimated to account for ~15% of the total mitochondrial matrix proteins. A decrease in aconitase activity is likely to affect the overall turnover efficiency of the citric acid cycle. In a recent study, mitochondrial aconitase was found to be the main target of an age-related increase in protein carbonylation in the mitochondrial matrix (34).

*1.1.4.2.3 Carnitine Palmitoyltransferase-1* Carnitine palmitoyltransferase-1, also known as carnitine acyltransferase-1, is a mitochondrial enzyme. In muscle and other nonliver tissues, this enzyme is associated with the outer mitochondrial membrane and mediates the transport of long-chain fatty acids across the membrane by binding them to carnitine (396). Liu et al. (397) found age-associated loss of binding affinity and activity of carnitine acetyltransferase and suggested two plausible mechanisms that could account for this loss: (i) adduct formation with aldehydic products of LPO or (ii) oxidation of the protein either directly by oxidants or by MCO. Beyond the latter mechanism, adduction was proposed to be more likely.

***1.1.4.3 Cytochrome P450 Enzymes*** CYP450 enzymes mostly take important place in drug toxicity. Elderly people consume 33% of all prescription drugs. Since the elderly are the most medicated segment of society they are presumed to be more vulnerable to drug toxicity and side effects. A number of human studies have demonstrated an age-related decline in the clearance of drugs undergoing biotransformation by CYP450 enzymes. Wauthier et al. (398) studied the influence of aging on the intrinsic capacity of rat liver CYP450 enzymes to metabolize xenobiotics. The functionality of CYP2E1 was found to be decreased. The decrease in CYP2E1 activity in rats after 11 months

was thought to be due to posttranslational modifications of CYP2E1 proteins. Among such covalent modifications, protein phosphorylation represents a rapid way to inactivate CYP2E1 (399).

***1.1.4.4 Protein Oxidation in the Nucleus and Chromatin*** At the first level of chromatin formation from DNA in eukaryotes, some 200 base pairs of DNA are forming a set with two of each of the histones H2A, H2B, H3, and H4. The structure formed is called nucleosome. It seems that disulfide (S-S) bonds appear to play a role in maintaining such a condensed structure of the chromatin complex. However, age-related alterations in chromatin were tested (400) and revealed that in chromatin, disulfide bonds appear to play a role in the linkage of regions of DNA with this nuclear protein skeleton structure.

*1.1.4.4.1 Histone Modification* Histones are the major protein components of chromatin. In nuclear oxidative damage, in addition to the DNA oxidation, protein oxidation also takes an important place, mainly targeting histone proteins. Nucleosomal histones protect DNA from free radical-mediated damage (401), and histone detachment and reattachment are closely connected with transcription and replication processes as with DNA repair and, therefore, require functionally intact histones. Oxidatively damaged histones are able to cross-link with DNA and would impair the detachment-reassembly process (402). Because of the long life span and low turnover rate of histones, proteolytic reactions are required to be highly selective and well regulated. Histones are subject to posttranslational modifications by enzymes, primarily on their N-terminal tails, but also in their globular domains. Poly(ADP-ribosyl)ation is the most dramatic posttranslational modification of histones in nuclei as well as in nucleosomes (403, 404). The well-established poly(ADP-ribosyl)ation of histones in response to DNA damage strongly suggests that poly (ADP-ribose) polymerase (PARP) plays an important role in DNA repair when DNA is structured in chromatin (405). Additionally, nucleosomal histones are known to protect DNA from free radical-mediated damage (406) and are susceptible to oxidative damage *in vitro* (401). It is therefore necessary to efficiently degrade oxidatively damaged histones to maintain genomic integrity.

Protein-bound reactive species on a histone or some other DNA BiP might be in a sufficiently close position to DNA to generate DNA damage. In this way, protein damage by radicals could be linked to mutations (45).

Zhao et al. (407) showed that 30- to 60-min exposure of cells to 100 or 200 μM $H_2O_2$ led to an increase in the level of H2A phosphorylation. Detection of DNA damage, indirectly, by immunocytochemical assessment of histone H2A phosphorylation offers much greater sensitivity compared with other methods for the determination of double strand breaks. However, it was observed that in untreated normal cells as well as in the cells of various tumor lines, a fraction of histone H2A molecules constantly undergoes

phosphorylation. The level of this constitutive H2A phosphorylation (CHP) was seen to vary depending on the cell type (line) as well as on the cell cycle phase, being the highest in S and G2M cells (408, 409).

Cervantes-Laurean et al. (410) investigated the nuclear protein targets of glycoxidation and cellular responses in cultured human keratinocytes exposed to glyoxal (GO) as a model toxicant. The concentration of GO (10 mM) used in this model was likely to be significantly lowered due to the nonenzymatic and enzymatic degradation of GO before it reaches the nuclei as reported by others (411). They observed that histones are modified by GO; this is in agreement with prior studies showing that oxidative damage caused the formation of carbonyl and fluorescent adducts in histones (401, 412). They observed that all histones were modified, with histone H1 showing the highest degree of modification on a protein basis, reflecting the higher lysine content of this histone. While histone H1 was degraded at a fast rate of degradation during 48 h after GO treatment, the rate for core histones was biphasic; the initial fast rate of degradation during the first 24 h was followed by slower rate of degradation between 24 and 48 h. This suggests that accumulation of CML-modified histones 72 h after GO treatment may be due to accumulation of modified core histones. A similar susceptibility toward damage was found by measuring carbonyl groups in histones after stressing cells with hydrogen peroxide (401, 413). CML-adduct formation on histones may have important implications in terms of histone function and genomic integrity, since this modification alters the ionic charge on histones. The carboxymethyl moiety of CML places a negative charge on the lysine residue, which replaces a positive charge on the α-amino group normally present in lysine residues. This is potentially important since CML modifications of lysine located in the N-terminal histone tails are the site of a number of posttranslational modifications that regulate chromatin function. These modifications include acetylation, phosphorylation, methylation, poly(ADP-ribosyl)ation, and ubiquitination (414), all of which are involved in regulatory functions in the processing of genetic information. Therefore, chronic accumulation of CML modifications could cause epigenetic changes that may alter gene expression. Thus, it seems reasonable to postulate the nucleus should have a more resilient proteasomal activity than other cellular compartments. In this study, modified histone adducts were found to be slowly degraded in keratinocytes following treatment with GO. To investigate the involvement of the proteasome in the degradation of histone adducts, keratinocytes were coincubated with GO and a proteasome inhibitor. The accumulation of histone-CML adducts was consistent with a role of the nuclear proteasome in degrading CML-modified histones.

Poly(ADP-ribosyl)ation was shown to decrease, causing a loss in nuclear proteasomal activity during aging. Furthermore, results showed that this decline in the PARP-1-mediated proteasomal activation is due to a decline in the expression and activity of PARP-1 in senescent fibroblasts. *In vivo* results were also supportive because the protein expression level of PARP-1

is significantly lower in the skin of aged donors compared with that of young ones (415).

***1.1.4.5 Protein Oxidation in the Endoplasmic Reticulum*** The ER is a focal point for intracellular protein assembly. Within the lumen of the ER, proteins are folded, posttranslationally modified, and assembled into protein complexes before they are exported to the cytoplasm, other organelles, the cell surface, or secreted from the cell altogether (416–418). Within the ER, PDI and immunoglobulin heavy-chain BiP belong to a cadre of chaperones and other enzymes that catalyze the proper folding and assembly of proteins. BiP is a member of the HSP70 chaperone family (419) and interacts with newly synthesized polypeptides through a C-terminal binding domain that preferentially interacts with linear stretches of amino acids that contain alternating hydrophobic and aromatic residues (420). BiP also plays a central role in the unfolded protein stress response (UPR) (421). BiP interacts with a variety of other chaperones and folding enzymes, participating in one of the major chaperone complexes within the ER (422). PDI is a disulfide isomerase that mediates the oxidative folding and unfolding of polypeptides within the ER (423, 424). It catalyzes the disulfide bond isomerization of a variety of substrates *in vitro* (425, 426). In the cell, however, PDI is directly oxidized through the Ero-1 pathway and functions as a sulfhydryl oxidase, catalyzing disulfide bond formation for polypeptide substrates in the process of folding into native structures (427). PDI also functions in the large network of ER chaperones that include BiP, Grp94, ERp72, and other folding enzymes (422). The concerted activities of these proteins are required for ER protein quality control (416).

The accumulation of misfolded proteins is a hallmark of the phenotype of aged tissues and is thought to be a causative factor in a variety of age-associated diseases such as Parkinson's disease and AD (428). Recent reports have emphasized that the ER is a major target for oxidative stress (427, 429, 430), and Rabek et al. have previously shown that PDI and BiP are oxidatively modified by carbonylation in aged mouse liver (431). The oxidizing environment of the ER makes resident proteins potential targets for ROS (427, 429, 430). It was previously shown that levels of carbonylated PDI and BiP are much higher in livers of aged mice compared with young mice. Additionally, several recent studies have shown that BiP and PDI protein levels decrease with age, further suggesting that chaperone dysfunction could be a principle cause of increased protein misfolding and accumulation of oxidatively modified proteins, a physiological basis for the progression of aging characteristics (432, 433). Nuss et al. (434) have shown that increase in age-related oxidative modification of PDI and BiP correlates to a decrease in their chaperone-like activities, thus suggesting a decline in tissue function due to ER dysfunction. Their results suggest that the activities of both chaperone proteins are altered by carbonylation. In addition to PDI and BiP, the loss of chaperone activity of many other folding enzymes such as ERp55, ERp57, ERp72, and calnexin

has been reported to decrease with age (432). They suggest that decreased ER chaperone activity and increased folding stress is one of the underlying mechanisms of aging.

Disulfide bond formation is an essential component of the protein folding process, and disulfide bonds are required for structural stability, enzymatic function, and regulation of protein activity (435). The catalytic events involving the oxidation, reduction, and isomerization of disulfide bonds take place in the ER. During protein oxidation, PDI introduces native disulfide bonds into substrate proteins and is reoxidized by the Ero proteins (Ero1p in yeast, Ero1α and Ero1β in humans) (436). In humans, PDI also contributes to collagen biosynthesis as a component of the prolyl-4-hydroxylase complex (437) and can act as a component of the ER degradation machinery, particularly with respect to the unfolding and retrotranslocation of toxins (438, 439).

Gadd153, also known as CHOP, is a leucine zipper transcription factor that is present at low levels under normal conditions but is robustly expressed in response to oxidative stress (440, 441). Gadd153 was originally identified based on its induction following treatment of cells with growth-arresting and DNA-damaging agents, though induced expression of the gene has also been strongly tied to perturbation of homeostasis in the ER.

As mentioned, proteins destined for transport to the cell membrane or to the cell exterior are synthesized in the ER and then are extensively modified by glycosylation and the addition of disulfide bonds. It is in the lumen of the ER, which provides a unique environment for protein folding, that proteins assume their mature, tertiary conformation. Disruption of homeostasis in the ER, which can occur, for example, as a result of nutrient deprivation or alteration of the organelle's calcium-rich oxidizing environment, can have devastating effects on the cell. Protein misfolding compromises cell function because essential polypeptides never exit the ER and are thus unable to perform their normal roles (442, 443). Additionally, accumulation of misfolded proteins in the ER triggers a unique signaling cascade referred to as the UPR. In the mammalian UPR, a signal is transduced from the stressed ER to the nucleus, where transcription of a number of genes, including Gadd153 and genes encoding ER resident proteins such as the glucose-regulated proteins (Grp genes), is activated (444, 445). The Grps function as chaperones that guide proteins through the folding process, and their upregulation in response to ER stress increases the cell's capacity to cope with the accumulation of immature, misfolded proteins in the ER. Indeed, if Grp78 induction is prevented, cell survival diminishes greatly following treatment with agents that stress the ER (446, 447). Following induction of the UPR, the kinetics of Gadd153 induction parallel exactly those seen for Grp78 (441). However, the effect of upregulating Gadd153 in response to protein misfolding is much less intuitive than the effect of upregulating expression of ER chaperones, and few studies have expressly addressed what function Gadd153 has in the ER stress response.

***1.1.4.6 Protein Oxidation in Peroxisomes*** Peroxisomal function declines with age (448), including the activity of catalase (449). Recently, peroxisome senescence seems to be related to the peroxisomal targeting signal protein import (450). The lack of peroxisomal carnitine acetyltransferase (CarT) in the nematode *Caenorhabditis elegans* has been shown to cause a progeric phenotype (451). Mi et al. (452) have isolated peroxisome-enriched fractions from two mouse tissues and found age-related peroxisomal dysfunctions. Peroxisome aging was initially investigated using a rat liver model. These studies documented age-related differences in peroxisomal enzyme activities and overall organelle function (453). Important follow-up work by Badr and colleagues confirmed the reduced activities of peroxisomal β-oxidation enzymes and catalase in aging rat liver, and suggested that with respect to the former, diminished levels of the peroxisome proliferator-activated receptor α (PPARα)-binding partner, retinoid X receptor α (RXRα), may be responsible (454). The overwhelming majority of peroxisomal enzymes, including most oxidases which produce hydrogen peroxide, contain a carboxy-terminal tripeptide closely related or identical to serine–lysine–leucine (455). Called peroxisomal targeting signal 1 (PTS1), this sequence and residues just upstream determine the enzymes' capacities to be recognized by the import receptor Pex5p (456, 457). Most of the binding studies involve measurement of direct protein–protein interactions between receptor and ligand. Such interactions may very well be facilitated or modulated in the cell by molecular chaperones or other factors. HSP70 is one such factor—shown to regulate Pex5p's binding to PTS1 (458)—and be a necessary component of (PTS1) protein import (459). Since its expression is reduced in aging (460), HSP70—or more accurately, its absence—may contribute to the processes associated with peroxisome senescence.

In particular, peroxisomes generate significant quantities of hydrogen peroxide, which under most conditions are metabolized by the organelle's resident catalase. Compromised catalase activity is associated with a large number of human pathologies, including osteoarthritis and degenerative joint disease, the initiation and progression of certain cancers, psoriasis and related (inflammatory) skin diseases, ischemia–reperfusion injury, neurodegenerative disorders, retinal disease, and type 2 diabetes, among others (461). The relative decline of peroxisomal catalase activity is seen in aging cells due to the progressive mistargeting of the molecule (450, 462–464), in specific human disorders due to instability of the enzyme or its message (465, 466), and in cells exposed to certain chemical agents/environmental toxicants due to inactivation of the antioxidant.

Koepke et al. (467) showed that 3-amino-1,2,4-triazole (3-AT) prematurely caused cellular aging and inactivation of catalase by 3-AT resulted in more oxidatively damaged protein in cells. This may be especially important when viewed in light of the fact that a number of low-grade environmental pollutants may also inactivate catalase, leading to chronic oxidative stress and potentiate the cellular aging phenomena described herein.

## 1.2 REVERSIBLE OXIDATIVE MODIFICATIONS

Some of the protein modifications are transiently reversible, and such reversal may provide reactivation. These protein modifications categorized as reversible are mainly repaired by enzymatic systems. Besides the mainly known reversible protein modifications, alterations involving residues on a protein surface may have less significant effects on protein conformation than alteration to internal residues, where oxidation may have a much more marked effect due to large increases in dipole moment (28). Moreover, Schiff base adducts formed upon reaction of an amine with a carbonyl are unstable (468), and so may be reversible inactivating lesions. Schiff base formation is, however, often followed rapidly by Amadori rearrangements (469), and hence the window for possible reversal is small. The thioether linkage formed by Michael reactions of protein-SH groups with 4-HNE is unlikely to be reversed biologically. During hypochlorite oxidation of LDL, in which protein is the preferential target, lysine-derived aldehydes seem to form protein cross-links that are initially reversible (470).

### 1.2.1 Methionine Sulfoxides and Methionine Modifications

Methionine (Met) is one of 20 common amino acids in proteins and is an important metabolite at the junction of methylation and transsulfuration pathways (471). However, this sulfur-containing amino acid is susceptible to oxidation by almost all forms of ROS, under conditions of oxidative stress (472). Depending on the nature of the oxidizing species, methionine may undergo a two-electron oxidation to methionine sulfoxide or one-electron oxidation to methionine radical cations. The one-electron oxidation of Met yields sulfide radical cations. These are highly unstable species which are converted into a series of intermediates and products, of which several would represent irreversible posttranslational protein modifications (473). Methionine radical cations will enter predominantly irreversible reaction channels, which ultimately yield carbon-centered and/or peroxyl radicals. These may become starting points for chain reactions of protein oxidation.

Methionine (Met) residues of proteins are readily oxidized by ROS to a mixture of *S*- and *R*-stereoisomers of methionine sulfoxide (MetSO) (Fig. 1.18, reaction 43). The *S*-isomer is often referred to as MetA and the *R*-isomer is referred to as MetB. Met oxidation may affect protein structure and function, and MetSO levels are known to increase under stress, in disease, and during aging (474–476). However, unlike oxidation of other amino acid residues (except Cys), oxidation of Met to MetSO is reversible. MetSO can be reduced back to Met by the action of reductases (MSR-A and MSR-B) that can catalyze the reduction of the *S*- and *R*-isomers of MetSO, respectively, back to Met. Both enzymes utilize Trx [$Th(SH)_2$] as a source of reducing equivalents (Fig. 1.18, reaction 44). MSR-A has a Cys at the catalytic site and, in most organisms, MSR-B has a selenocysteine at the catalytic site. Moreover, in the

| | |
|---|---|
| $Met + ROS \rightarrow MetO + products$ | **Reaction 43** |
| $MetO + Th(SH)_2 \rightarrow Met + Th(S\text{-}S) + H_2O$ | **Reaction 44** |
| $Th(SS) + NADPH + H^+ \rightarrow Th(SH)_2 + NADP^+$ | **Reaction 45** |
| $NADPH + H^+ + ROS \rightarrow NADP^+ + H_2O + products$ | **Reaction 46** |

**FIG. 1.18** Methionine residues of proteins can be readily oxidized by different ROS to both *S*- and *R*-stereoisomers of methionine sulfoxide (MetO) (**reaction 43**). MetO can be again reduced back to methionine by the reductases MSR-A and MSR-B that catalyze the reduction of the *S*- and *R*-isomers. Both enzymes use Trx ($Th(SH)_2$) as reductant (**reaction 44**). In the presence of NADPH, the oxidized form of Trx (ThS-S) can be reduced via the enzyme Trx reductase (**reaction 45**). Coupling of the **reactions 43–45** results in **reaction 46**, providing an NADPH dependent mechanism of ROS scavenging.

presence of NADPH, the oxidized form of Trx (ThS-S) can be converted back to its reduced form by the enzyme Trx reductase (Fig. 1.18, reaction 45). The coupling of reactions 1–3 leads to the overall reaction 46 (Fig. 1.18), and thus provides a mechanism for conversion of ROS for the scavenging of ROS species. Mutations leading to a decrease in MSR activities are associated with a decrease in resistance to oxidative stress and to a shortening of the maximal life span, whereas mutations leading to overproduction of MSR activities lead to an increase in resistance to oxidative stress and a significant extended life span (29).

There are bulk of studies concerning methionine oxidation and repair of this process. CaM–plasma membrane $Ca^{2+}$ ATPase activity is downregulated by the oxidation of the two specific CaM methionines out of a total of seven (477), and methionine sulfoxide reductase reductively functions to upregulate the enzyme system (478).

$\alpha_1$-Proteinase inhibitor is inactivated on oxidation of a susceptible methionine residue, and $H_2O_2$ inactivates a neutrophil cytosolic serine proteinase inhibitor (serpin), possibly via a similar process (479). In some cases, these reactions of $H_2O_2$ and methionine may be nucleophilic (molecular) rather than radical mediated. The proteinase subtilisin is dependent on a methionine residue both for its activity and for its susceptibility to $H_2O_2$ *in vitro*; replacement of this residue by site-directed mutagenesis decreases inactivation of the enzyme by $H_2O_2$ (480).

t-Butyl hydroperoxide behaves like $H_2O_2$, but only oxidizes exposed methionines (481), presumably because its increased steric bulk limits access. Treatment of isolated human erythrocyte GAPDH and glycophorin with ozone also leads to the loss of both methionines. In the latter, no other amino acids are oxidized, and the reactions are probably nucleophilic (482). A number of neutrophil neutral proteinase inhibitors, isolated *E. coli* GS, and BSA, when exposed to ozone, also show methionine oxidation and lesser oxidation of

histidine and aromatic residues (67). Further oxidation products of methionine sulfoxide (e.g., the sulfone (483)) cannot be reversed by the reductase.

Amino acid analysis of oxidized retinoblastoma protein (pRB) showed methionine oxidation to methionine sulfoxide. The monochloramine ($NH_2Cl$)-treated Jurkat cell proteins also showed a decrease in methionine. These observations suggested that direct pRB oxidation was the major cause of $NH_2Cl$-induced cell cycle arrest (484).

The effect of hydrogen peroxide exposure upon GS from *E. coli* was studied by Levine et al. (485) as an *in vitro* model system. Eight of the 16 methionine residues was oxidized with little effect on activity. The oxidizable methionine residues were found to be relatively surface exposed while the intact residues were generally buried within the core of the protein. Further, the susceptible residues were physically arranged in an array which guarded the entrance to the active site.

Based on the reactions of the oxidation and repair of methionine, it was proposed that the oxidation–reduction of Met residues of proteins may serve an important antioxidant function (486). The importance of MSR in aging was highlighted by results of studies showing that: there is an age-related increase in the surface hydrophobicity of rat liver proteins (487), which may be due in part to the fact that ROS-mediated oxidation of methionine residues in proteins leads to an increase in surface hydrophobicity (488, 489); there is an age-related increase in orthotyrosine (o-tyr) and MetSO in human skin collagen (490); and overexpression of *S*-MSR in *Drosophila* leads to an almost doubling of the maximal life span (491). This effect of MSR on life expectancy is supported by studies demonstrated that mutant strains of yeast (492), bacteria (493–495), and mice (496) that lack the MSR-A gene are more sensitive to oxidative stress or live shorter. Moreover, overexpression of the MSR-A gene in yeast (497), neuronal PC-12 cells (498), human T cells (497), and *Drosophila* (491) leads to increased resistance to oxidative stress. The singular importance of methionine oxidation in aging is highlighted by the following findings:

(i) The level of MSR-A in various rat tissues declines with age (487, 499) and in some age-related diseases, that is, AD (500), emphysema (especially in cigarette smokers) (62, 501), bronchitis (502), and Parkinson's disease (503, 504).

(ii) Mutations leading to a loss of MSR-A activity in mice lead to a 40% decrease in the maximum life span (496).

(iii) Overexpression of MSR-A in flies leads to a nearly doubling of the life span (491).

(iv) There is a slight age-related increase in the level of MetSO in the α-crystallins (505).

(v) The MetSO/Met ratio in acetic insoluble proteins from trabecular meshwork increases from 10 to 40 during aging, over the range of 10–80 years (506).

(vi) There is a three- to fourfold increase in the level of MetSO in human skin collagen, over the range of 10–80 years (490).

(vii) The level of MetSO from young, mature, and senescent human erythrocytes (separated by density gradient sedimentation) is about 50% and 60% higher, respectively, than in the young erythrocytes (349, 507).

(viii) It is reported that there is a progressive age-related increase in the oxidation of Met residues in Fisher 344 rat brain CaM, over the range of 6–27 months, which is associated with a loss in the ability of the protein to regulate plasma membrane ATP hydrolysis and ATP-dependent $Ca^{2+}$ transport (508, 509). However, more recent studies demonstrate that oxidation of CaM does not occur in currently available strains of rats, raised under present husbandry conditions.

(ix) MSR-A and MSR-B (hCBS-1) gene expression is downregulated during replicative senescence of WI-38 human fibroblasts (510).

(x) Evidence that enhanced oxidation of protein by neutrophil-generated ROS contributes to the development of chronic and acute bronchitis is supported by the observation that these abnormalities are associated with substantial increases in the neutrophil content and in the MetSO/Met ratio of bronchoalveolar lavage fluid of individuals suffering from these disorders (502). Furthermore, during *E. coli* senescence, induced by nitrogen starvation, there is a 1.5- to 2-fold increase in the levels of MetSO and carbonyl content of proteins. There are higher levels of MetSO in the brain regions compared to other organs, with the exception of the cerebellum, which has relatively high activity of MSR-A (511).

Oxidatively sensitive calcium regulatory proteins that modulate the activity of calcium channels and pumps include CaM and phospholamban, which contain sensitive methionines whose oxidation to their corresponding methionine sulfoxides results in a disruption of secondary structure that modulates the activity of the bound complex (512). In the course of aging or under conditions of oxidative stress, methionine residues of CaM undergo oxidation, leading to loss of biological activity of the protein. The calculated changes in affinity for calcium and for the target peptide show opposing trends. Oxidation at position 144 is predicted to enhance peptide binding and weaken calcium binding, whereas oxidation at 145 weakens peptide binding and enhances affinity for calcium (513). An increasing number of studies suggest that oxidative damage to proteins can be correlated with the loss of calcium homeostasis in a number of diseases and aging (362, 514, 515). In the case of CaM, oxidative modifications of multiple methionines to sulfoxide have been identified from *in vivo* studies of aged rat brains (508) and *in vitro* experiments involving exposure to hydrogen peroxide or peroxynitrite (516). Oxidation significantly decreased the CaM affinity for binding calcium (517). CaM contains nine

methionines altogether, all of which are susceptible to oxidation in some degree. Likewise, the oxidation of Met20 in the switch region of phospholamban has been reported to be oxidized in human heart (518). The oxidation of methionines in CaM can also affect the activity of calcium pumps and channels to modulate the amplitude and duration of calcium signals. Boschek et al. (519) investigated the possible oxidation of CaM in skeletal muscle and its effect on the CaM-dependent regulation of the RyR1 calcium release channel. They found that approximately two methionines are oxidized in CaM from skeletal muscle. These results suggest that the oxidation of CaM can contribute to observed elevations in intracellular calcium levels in response to conditions of oxidative stress observed during biological aging. As a conclusion, the sensitivity of RyR1 channel activity to CaM oxidation may function as part of an adaptive cellular response that enhances the duration of calcium transients to promote enhanced contractility. On the other hand, Michaelis and colleagues reported the intriguing observation that about six of the nine methionine residues of rat brain CaM are oxidized to the sulfoxide in protein purified from old animals (508).

Different kinds of radicals may cause methionine oxidation. Diffusion-controlled addition of hydroxyl radical to the sulfur represents the first step in the reaction of $^{\bullet}OH$ with aliphatic sulfides, yielding hydroxysulfuranyl radicals (520). These hydroxysulfuranyl radicals are quite unstable, unless they can be stabilized via hydrogen bonding (521), and decompose either unimolecularly or in a proton-catalyzed process into sulfide radical cations and $^{\bullet}OH$ or $H_2O$, respectively (520). Originally, such intramolecular proton transfer was reported for the amino acid Met, where the initially formed hydroxysulfuranyl radical rapidly converts into a sulfur–nitrogen (S∴N) three-electron bonded complex, which is ultimately decomposed via electron transfer from the carboxylate group to yield $CO_2$ and an α-amino-substituted radical. An analogous mechanism also operates in peptides. For example, in the model peptide threonylmethionine (Thr-Met), hydroxysulfuranyl radicals at the Met sulfur decompose via proton transfer from the N-terminal amino (522), and this mechanism still operates when both amino acids are separated by up to four spacing glycine (Gly) residues, that is, in Thr-(Gly)4-Met (523). In these peptides, the intermediary (S∴N)-bonded radicals do not decarboxylate, but rather undergo homolytic cleavage of the $C_\alpha$–$C_\beta$ bond of Thr, yielding acetaldehyde.

An important characteristic of sulfide radical cations already apparent from these reactions is their tendency to associate with electron-rich heteroatoms such as N, O, S, P, and Se. In free Met and the Thr-(Gly)n-Met model peptides, the intermediary (S∴N)-bonded species spontaneously decompose into either $CO_2$ or acetaldehyde, respectively. In N-acetylated model peptides, such as N-Ac-Met-$NH_2$, N-Ac-Gly-Met-Gly, and N-Ac-(Gly)3-Met-(Gly)3, hydroxysulfuranyl radicals convert into sulfur–oxygen (S-O) three-electron bonded species formed with the carbonyl oxygen of the peptide bond located either at the N- or the C-terminal of the Met residue (473).

Enzymatically generated dioxetanes provide an established entry into electronically excited triplet species *in vivo*, predominantly triplet aldehydes and ketones (524). Moreover, triplet excited species may be generated photochemically in light-exposed tissue. Model studies with excited triplet benzophenones have given a detailed picture on the oxidation mechanisms of Met, Met-containing peptides, and a series of aliphatic sulfides (525). A large body of experimental and theoretical work on the reaction of singlet oxygen with sulfides is available (526). Initially, the reaction of singlet oxygen with a sulfide generates a persulfoxide intermediate. The chemistry of the persulfoxide is highly dependent on the nature of the sulfide and the solvent. In water, a rapid hydration of the persulfoxide is most likely, where the product hydroperoxysulfurane will either oxidize a second sulfide or eliminate hydrogen peroxide. The reaction of singlet oxygen with free Met yields azasulfonium salts (527), which subsequently hydrolyze to MetSO. Interestingly, this mechanism does not operate with the dipeptide Met-Met, which undergoes a clean oxidation to the disulfoxide, MetSO-MetSO (528). Peroxynitrite ($ONOO^-$) and peroxynitrous acid (ONOOH) also oxidize Met to MetSO (529).

Aβ plays a key role in the pathogenesis of AD and contains a Met residue at position 35 in its C-terminal domain, which is critical for neurotoxicity, aggregation, and free-radical/ROS formation initiated by the peptide. However, the ultimate generation of some MetSO on Aβ appears secondary compared with the important fact that Met likely serves as an electron donor for the reduction of Aβ-bound $Cu^{II}$ to $Cu^{I}$. Stoichiometrically, such a process requires the one-electron oxidation of Met to its radical cation, $MetS^{\bullet+}$, and it appears that specific chemical properties of $MetS^{\bullet+}$ play an important role in the processes underlying Aβ neurotoxicity and free-radical generation (530).

In α1-antiproteinase, oxidation of $Met^{358}$ to methionine sulfoxide destroys the antiproteinase activity, presumably by interfering with complex formation with the target proteinase (485). However, the thiol ether is not required for interaction since replacement of the methionine with valine gives a fully active antiproteinase (531). More recently, the oxidative inactivation of $\alpha_2$-macroglobulin has been studied in detail and also gave results consistent with the hypothesis (532). This antiproteinase loses activity when exposed to activated neutrophils, or to a model system consisting of chloramine. The consumption of eight equivalents of chloramine caused the oxidation of eight methionine residues to methionine sulfoxide. Continued exposure caused oxidation of six additional residues of methionine and of a single tryptophan residue. The fractional loss of the tryptophan residue matched the fractional inactivation of the $\alpha_2$-macroglobulin. These results are consistent with the suggestion that oxidation of methionine residues scavenge oxidants which could otherwise attack the tryptophan residue that is essential to function. Susceptibility generally correlates with the surface exposure of the residue, although residues near the methionine can modulate their susceptibility (533). Hsu and colleagues (534) studied oxidation by hydrogen peroxide of recombinant human stem cell factor, which contains five methionine residues. The two

surface-exposed residues, $Met^{1}$ and $Met^{159}$, were readily oxidized, but with negligible effects on biological activity. $Met^{27}$ was oxidized at about one-third the rate of the rapidly oxidized residues, again with little effect on activity. The remaining two residues, $Met^{36}$ and $Met^{48}$, were much less susceptible to oxidation and modification of either residue was accompanied by a substantial loss of biological activity. Similarly, Gitlin and colleagues established that oxidation of $Met^{111}$ in interferon α-2b did not alter its biological activity (535). Nabuchi and coauthors reported studies on hydrogen peroxide-mediated oxidation of the two methionine residues present in human parathyroid hormone (536). Oxidation of $Met^{8}$ slightly reduced biological activity while oxidation of $Met^{18}$ substantially reduced activity. Keck showed that two surface-exposed methionine residues of interferon or three methionine residues of tissue plasminogen activation could be oxidized without loss of biological activity (481). A similar result with keratinocyte growth factor has been summarized recently (537). While solvent-exposed methionine residues are likely to protect from environmentally proximate oxidizing agents, residues in or near active sites may protect enzymes from "autoxidation" by substrates or cofactors. For example, oxidation of a single methionine in rabbit 15-lipoxygenase was known to be mediated by substrates or products, and the appearance of the methionine sulfoxide had been correlated with loss of catalytic activity. However, the studies of Gan and colleagues established that replacement of the methionine residue by leucine did not prevent inactivation by substrates, demonstrating that formation of methionine sulfoxide was not the cause of inactivation (538). It was suggested that oxidation of the active site methionine may actually retard the inactivation of the lipoxygenase. It is notable that a significant number of methionine residues in GS may be oxidized without an increase in surface hydrophobicity or proteolytic susceptibility. The methionine sulfoxide content of human skin collagen increases from about 4% in the young to about 12% of methionine at age 80 (490).

Ciorba et al. (539) showed that oxidation of a methionine residue in a voltage-dependent potassium channel modulates its inactivation. When this methionine residue is oxidized to methionine sulfoxide, the inactivation is disrupted, and it is reversed by coexpression with peptide MSR. The results suggest that oxidation and reduction of methionine could play a dynamic role in the cellular signal transduction process in a variety of systems (62).

### 1.2.2 Cysteine Modifications and Disulfide Bond Formation

Oxidation of two Cys residues of the same protein or of two different proteins can lead to the formation of intra- or interdisulfide cross-linked derivatives (Fig. 1.19) (29). The formation of disulfide bonds can be reversed by glutaredoxin 1 and by Trx (540).

Cys, homocysteine (Hcys), GSH, and cysteinylglycine (CysGly) are the most abundant low-molecular-mass sulfhydryls (LMM-SHs) occurring in the extracellular milieu and, together with albumin, represent almost all the thiols in

$$\text{Protein}^1\text{-SH} + \text{Protein}^2\text{-SH} + H_2O_2 \rightarrow \text{Protein}^1\text{-S-S-Protein}^2 + 2H_2O \qquad \textbf{Reaction 47}$$

**FIG. 1.19** An exposure of the protein OxyR to hydrogen peroxide ($H_2O_2$) converts $Cys_{199}$ to a sulfenic acid derivative that forms an intramolecular disulfide bond with $Cys_{208}$.

plasma. All these molecules are metabolically interrelated and are important in determining the redox environment and free-radical interactions (541, 542). Cys is unstable and toxic at high concentration; however, cells and tissues have an absolute requirement for it, and plasma GSH seems to be a critical source for maintaining steady Cys availability. Cys may also derive from intracellular stores of various tissues (e.g., skeletal muscle during starvation) that can deliver it to the plasma; the other main source is diet (543). Methionine can also serve as a source of Cys because it can be converted to this amino acid through the transsulfuration pathway. Hcys does not derive directly from GSH but is an intermediate in the transformation of methionine into Cys, and is probably delivered by all those cells capable of performing the transsulfuration pathway reactions (544). Human plasma contains GSH, Cys, CysGly, and Hcys in the 0.1- to 20-μM range (541). These thiols can also be found in disulfide forms, both as low-molecular-mass disulfides (LMM-SS) and as protein/LMM-SH mixed disulfides. Usually, disulfide forms (with the exception of glutathione disulfide [GSSG]) are more concentrated than the respective thiol. Considering the total concentration for each thiol species (i.e., reduced + disulfide forms), Cys is usually present at the highest concentration, with CysGly, Hcys, and GSH (545). Additionally, the ratio Cys/cystine is significantly shifted to the oxidized species with respect to the ratio GSH/GSSG, suggesting that these may represent two pools with distinct regulation and significance (542).

Giustarini et al. 2006 (546) have focused on plasma thiols to assess whether during aging there is a shift in the thiol/disulfide balance, and found evidence for the age-related variation in the relative percentages of all thiol forms (i.e., reduced, disulfide, and protein-mixed disulfides). A study was published in which all redox forms of plasma thiols have been investigated in relation to aging in the age range of 21–92 years. The data show that a strong inverse correlation exists between aging and levels of CysGly and GSH, whereas neither their disulfides nor tGSH and tCysGly changed. Conversely, direct correlations were found between the disulfide forms of Cys and Hcys and age. As expected, the concentration of protein SH groups decreased with age, as a consequence of the increased levels of mixed disulfides with Cys and Hcys, and the decline of the plasma concentration of albumin that takes place in elderly persons (547). It has also been observed in previous work that an oxidative shift of Cys and GSH toward disulfide forms occurs in human plasma in elderly persons (543, 548).

| | |
|---|---|
| $Protein^1\text{-}S\text{-}S\text{-}Protein^2 + 2GSH + H_2O_2 \rightarrow Protein^1 + Protein^2 + GSSG$ | **Reaction 48** |
| $Protein^1\text{-}S\text{-}S\text{-}Protein^2 + Th(SH)_2 \rightarrow Protein^1 + Protein^2 + Th(SS)$ | **Reaction 49** |
| $Protein\text{-}SSG + GSH \rightarrow Protein\text{-}SH + GSSG$ | **Reaction 50** |

**FIG. 1.20** Thiol transferases are able to repair disulfide derivatives, by catalyzing reactions between glutathione (GSH) or Trx ($Th(SH)_2$) to restore the sulfhydryl groups of a protein (**reactions 48–50**).

Cys residues of proteins are particularly susceptible to oxidation by ROS (62). In contrast to other ROS-mediated oxidations, oxidation of the sulfur amino acids is reversible. Oxidation of Cys sulfhydryl groups of proteins leads to the production of either intramolecular ($P^1SSP^1$) or intermolecular ($P^1SSP^2$) protein cross-linked derivatives, and reactions with GSH yields the mixed disulfide (PSSG). These disulfide derivatives can be repaired by disulfide exchange reactions catalyzed by thiol transferases that catalyze reactions between GSH and Trx [$Th(SH)_2$] to regenerate the protein sulfhydryl groups (Fig. 1.20).

Both GSSG and Th(SS) will be reduced back to their sulfhydryl forms by reductases (549). Cys residues function in the catalytic cycle of many enzymes, and they form disulfide bonds which contribute to protein structure. Sulfur exists stably in multiple oxidation states, which makes it a versatile component in biological systems. The most highly active and most reduced form of sulfur in biomolecules is the thiol (-SH), present in the amino acid Cys. Cys is present in the active site of many proteins and in protein motifs that function in enzyme regulation, protein trafficking, control of gene expression, and receptor signaling. The body's supply of Cys is from protein in the diet and through the transsulfuration of the essential dietary amino acid, Met. Accumulating data suggest that reversible oxidations of sulfur residues are common and fundamentally important in the control of cell functions (543).

Functional consequences of -SH loss include protein misfolding, catalytic inactivation, decreased antioxidative capacity, and loss of certain specific functions such as binding of heavy metals and sulfur-containing amino acids by albumin, among others. Age-associated losses in protein -SH content have been reported in a variety of tissues and species, including homogenates of brain, heart, skeletal muscle, and kidney of rodents and houseflies (27).

Conversion of -SH groups into disulfides and other oxidized species (e.g., oxyacids) is one of the earliest observable events during the radical-mediated oxidation of proteins, with the ratio of disulfide to oxygenated materials depending on the environment (44). Higher sulfides and persulfides may also be generated on proteins (550), resulting in conformational changes.

Inactivation of enzymes by limited -SH oxidation has been studied, mainly with oxidized thiols (e.g., GSSG) as oxidants, so as to restrict the range of

reactions. Thus, treatment of aldose reductase with GSSG results in mixed disulfide formation, conformational changes, and inactivation of the enzyme, which can be reversed by GSH (551). Reversible S-thiolation has also been detected on proteins in cells exposed to radicals (552). Furthermore, the respiratory burst of human monocytes results in rapid and reversible S-thiolation of a number of cytosolic proteins (553). Thus, biological thiols, such as GSH and Cys, can influence oxidant-induced protein inactivation either by direct reaction with the radicals or indirectly by forming reversible bonds with (normally free) thiols on the proteins. The latter should be reversible; it is also an essential process in *de novo* protein folding and in the maintenance of conformation.

### 1.2.3 Surface Hydrophobicity Modifications

An increased surface hydrophobicity of oxidized proteins is the key factor in their proteolytic recognition (15). Hydrophobicity was measured by using the binding of 8-anilino-1-naphthalene-sulfonic acid by Chao et al. (488). It was determined that the hydrophobicity of liver proteins of 24-month-old rats was some 15% higher, compared with 2-month-old animals. Interestingly, the surface hydrophobicity correlated with an increase in methionine sulfoxide and dityrosine. The oxidation of GS by an MCO system (554) led to an increase of surface hydrophobicity and susceptibility to proteolytic degradation. They reported that the surface hydrophobicity of rat liver proteins increases with animal age, and that *in vitro* exposure of rat liver proteins to an MCO system or to 2,2′-azobis(2-amidino-propane) dihydrochloride (AAPH) leads to an increase in surface hydrophobicity, protein carbonyl content, and conversion of methionine residues to methionine sulfoxide (MetSO) residues. In addition, treatment with AAPH, but not the MCO system, leads to oxidation of tryptophan residues, precipitation of some proteins, and formation of dityrosine derivatives. The increase in surface hydrophobicity correlated with an increase in dityrosine and MetSO formation.

## 1.3 IRREVERSIBLE OXIDATION PRODUCTS

Many protein oxidation products may be irreversible, and these oxidation products are mainly degraded by proteolytic systems. In the aging process, these products accumulate as a result of decrease in the proteolytic activities or overwhelming production of them. Following the exposure to oxidants, unfolding of the proteins may be irreversible. The other major component of irreversible protein oxidation is fragmentation, and when this is extensive it inevitably leads to a loss of conformation (44). The sizes of the fragments generated from BSA, hemoglobin, and myoglobin by radiolytic $^{\bullet}OH$ are compatible with fragmentation at proline residues in the protein backbone (555, 556). Indeed, proline (and histidine) is an important site of $^{\bullet}OH$ attack on

BSA, although the predicted product(s) from cleavage at this site could not be detected. Glycine residues also appear to be important in the fragmentation of calf skin collagen induced by a xanthine oxidase system (557). The cleavage is probably induced by $^{\bullet}OH$ in this system, as the primary $O_2^{\bullet-}$ produced is inactive in chain breakage. Of the N-terminal amino acids generated by fragmentation, 90% were glycine, even though the parent molecule contains only approximately 30% glycine. Selective fragmentation has also been observed with the apoB protein of LDL during either radiolysis or metal ion-catalyzed damage (558); it is difficult to assess the relevance of cleavage at proline or glycine in this case, due to difficulties in measuring fragment sizes. Using proteins that lack one of Tyr, Trp or His, Guptasarma et al. (559) demonstrated the importance of His in covalent protein cross-linking in the presence of $O_2$. Lys was also demonstrated to be involved; these cross-links may involve Schiff base derivatives.

### 1.3.1 Protein Oxidation and Enzymatic Posttranslational Modifications

Posttranslational protein modifications play an important role in the regulation of protein function through the modulation of protein structure, activity, turnover, localization, and the nature of protein–protein complexes (560). Today, more than 200 different posttranslational modifications are known (561), which are the result of both enzymatic and nonenzymatic processes. There is increasing evidence that posttranslational modifications of specific proteins accompany pathologic processes and biological aging. An important goal of global and targeted proteomic experiments must, therefore, be the identification and functional characterization of posttranslationally modified proteins *in vivo*, and to resolve the question whether such posttranslational modifications are mechanistically related (in contrast to merely being associated with) to a disease process or a specific phenotype of aging.

The redox-sensitive Cys residue in the structure of protein tyrosine phosphatases may be converted by GSH disulfide into a mixed disulfide with concomitant loss of catalytic activity (562). Cys sulfenic acids are highly reactive and are expected to react with GSH at its relatively high intracellular concentration. Therefore, it is reasonable to assume that ROS-induced oxidation will also lead to the rapid glutathionylation of the redox-sensitive Cys moiety. Glutamate is the major excitatory neurotransmitter of the central nervous system. NMDA-type glutamate receptors have been implicated in multiple physiological processes, including neuronal development (563). However, overstimulation of NMDA receptors can cause excessive $Ca^{2+}$ influx, free-radical generation, abnormal enzymatic activity, and thus contribute to a number of neurodegenerative diseases (564). A variety of chemical modifications that modulate NMDA receptor activity have been reported, including phosphorylation of tyrosine (565) and serine/threonine residues (566), redox modulation of disulfide bonds, and S-nitrosylation of free thiol groups on critical Cys residues (567, 568). It was shown that hypoxia enhances $^{\bullet}NO$

modulation of the NMDA receptor, resulting in increased attenuation of NMDA-evoked currents. Since Cys residues are the primary target for S-nitrosylation, the redox state of the NMDA receptor is critical to the efficacy of •NO modulation. NMDA receptors normally function in the brain where $pO_2$ levels are substantially lower than in ambient air. Under pathologically hypoxic conditions, S-nitrosylation of the NMDA receptor becomes even more important because progressively lower concentrations of •NO inhibit excessive NMDA receptor activity. A likely explanation for the enhancement of •NO inhibition of NMDA-evoked currents during hypoxia is the increase in free thiol groups that are available to react with •NO under these conditions (569).

### 1.3.2 Deamidation and Transamination

Transglutaminases (TGs) are $Ca^{2+}$-dependent enzymes that catalyze the deamidation and transamination of protein glutamine residues. While deamidation results in the formation of glutamic acid, transamination usually leads to γ-glutamyl-ε-lysine (GGEL) cross-links. Increasing evidence is accumulating that TG-dependent protein cross-linking plays an important role in oxidative stress (570) and some neurodegenerative diseases and aging. For example, the microtubule-associated protein tau represents a target for TG *in vitro* and *in vivo*, where, *in vitro*, TG-dependent cross-linking primarily affects protein domains located in close proximity to microtubule-binding domains (571). Increased TG activity appears to correlate with increased neuronal death in AD (572) and Huntington's disease (HD), as crossing HD R6/1 transgenic mice with TG knockout mice results in a large reduction of cell death (573). The exposure to TG of a four-repeat domain of human tau, τ4RD, human α-synuclein, and the N-terminal domain of the yeast prion protein Sup35, SupNM, abolished the tendency of these proteins to aggregate formation (574). This is most likely due to the formation of intramolecular GGEL cross-links, which may then reduce the conformational freedom of these polypeptides to aggregate intermolecularly en route to fibril formation.

Asparagines and, to a lesser extent, glutamines are prone to spontaneous deamidation where the amide function on the amino acid side chain is replaced by a carboxyl group (575). Following each deamidation event, there are four possible products, all of which alter the amino acid composition of the protein to some degree. Deamidation of asparagine residues will produce any one of the following—an L-aspartic acid, D-aspartic acid, L-iso-aspartic acid, and D-isoaspartic acid residue. The rates at which particular asparagine residues deamidate is strongly affected by adjacent amino acids, for example, asparagines with glycine, histidine, alanine, or serine on their carboxyl sides show a high deamidation incidence, with half-lives as short as 1 day (576).

Deamidation of glutamines is about 100 times slower than that of asparagine. As asparagine deamidation proceeds at a biologically significant rate, there is an enzyme present on most life forms that partially corrects this aberration. This is called protein isoaspartate methyltransferase (PIMT), whose

activity converts the isoform back to the normo-configuration; it does not, however, convert the carboxyl side chain back to an amide structure (577). Mice deficient in PIMT show intracellular accumulation of aberrant polypeptides, especially in their brains, and a decreased life span (578). Overproduction of PIMT has been shown to extend the life span of fruitflies but only when subjected to heat stress (579).

### 1.3.3 Protein Glycation and AGEs

Glucose has been implicated in the aging process by its ability to react nonenzymatically with proteins, especially those which are long-lived such as collagen to produce fluorescent and chromophoric adducts and cross-links (580). Glycation, a nonenzymatic reaction between a reducing sugar and a lysine residue, leads to the formation of protein AGEs such as $N^{\varepsilon}$-CML, $N^{\varepsilon}$-carboxyethyllysine (CEL), pentosidine, glyoxal lysine dimers (GOLDs), and methylglyoxal lysine dimers (MOLDs) (581). In the initial phase of this reaction, reducing sugars like glucose react with the free amino group of proteins by nucleophilic addition, resulting in the rapid formation of a Schiff base. This compound, in turn, can undergo rearrangement to form an Amadori product. Both the Schiff base and the Amadori rearrangement are reversible reactions, but the latter is more stabile. Reversibility as measured by the half-life of the Amadori product is difficult to assess due to the loss of the compound from simultaneously occurring sugar fragmentation, dehydration, and browning reactions (582). Since the reaction is the initial step in a complex series of reactions leading to the formation of brown nitrogenous polymeric compounds, the Amadori rearrangement is commonly referred as an early glycation product of the nonenzymatic browning or Maillard reaction pathway. Furosine is formed during acid hydrolysis through the cyclization of the carbon chain tail from the hexose sugar of the Amadori product, resulting in a 2-substituted furan ring-type structure (Fig. 1.21).

Glucose is the most abundant but least deleterious of the common metabolic sugars in humans; galactose and fructose are between four- and sevenfold more reactive. Most glycolytic intermediates are also much more reactive than glucose, especially the trioses glyceraldehyde- and dihydroxyacetonephosphates. The latter two are even more problematic because they can spontaneously generate methylglyoxal (MG), which is a highly reactive glycating agent (583, 584). Some MG is also generated metabolically from amino acids and lipids. MG has been implicated in age-related protein, lipid, and organelle dysfunction as well as a number of age-related pathologies including AD and diabetic secondary complications. In fact, MG can provoke many of the deleterious changes that accompany normal aging (formation of protein carbonyls groups and cross-linking, lipid and DNA damage, ROS, mitochondrial damage, and apoptosis). It is at least possible that the beneficial effects of dietary restriction and fasting may be explained in terms of suppression of both extracellular glucose and MG levels, compared with the *ad libitum*-fed condition

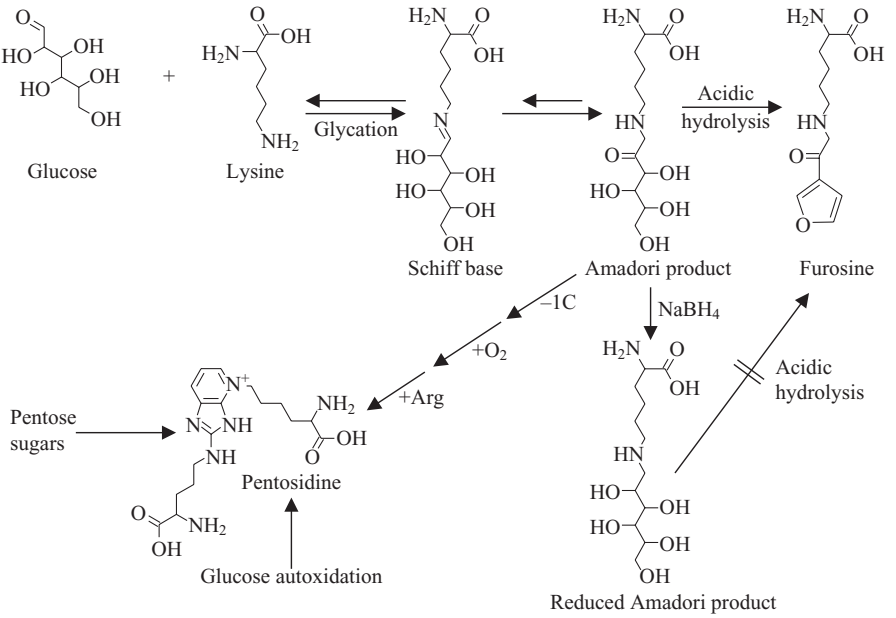
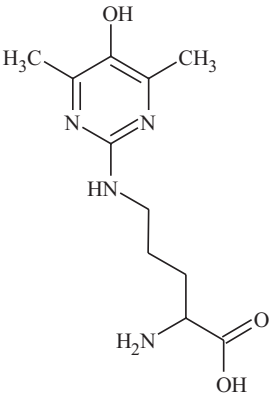



**FIG. 1.21** The formation of furosine and pentosidine from the Amadori product of glucose (according to Sell, Mech. Ageing Dev. 95: 81–99, 1997).

(585). The reaction of MG with proteins represents one pathway leading to the formation of AGEs, prominent protein modifications accompanying, for example, biological aging and diabetes. Proteomic studies identified a series of mitochondrial proteins as targets of MG-dependent modification in the diabetic rat kidney (586). MG forms various stable adducts with arginine residues such as $N^{\delta}$-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithin (MG-H1), $N^{\delta}$-[5-(2,3,4-trihydroxybutyl)-5-hydro-4- imidazolon-2-yl]ornithin, and argpyrimidine (Fig. 1.22). The inactivation of proteins through the accumulation of AGEs has been reported (587). The sensitivity of protein Arg residues toward MG *in vivo* may not only be defined by their chemical microenvironment (controlling accessibility and protonation equilibria) but also by the action of protein arginine methyl transferase (PRMT). The latter class of enzymes can transfer one and/or two methyl groups onto protein Arg residues, where dimethylation would prohibit the reaction with MG. Hence, dimethylation could protect protein Arg residues against the formation of MG-H1 (588), analogous to a mechanism characterized for human crystallins, where Cys methylation protects these proteins against oxidative modification/aggregation (589).

GO and arabinose are major autoxidation products of glucose; the former can form $N^{\varepsilon}$-CML and the latter can participate in the generation of the fluorescent pentosidine cross-links in proteins. Ascorbate and dicarbonyl sugars




Argpyrimidine

**FIG. 1.22** Molecular structure of argpyrimidine.

such as MG and 3-deoxyglucosone (3-DG), which are observed *in vivo*, may participate in autoxidative reactions contributing to browning. The protein-bound sugar Amadori intermediates are generally more readily autoxidized than free sugars, so the relative importance of autoxidation before and after protein binding may vary with the relative concentrations of the components. $N^{\varepsilon}$-CML and pentosidine accumulate *in vivo* with aging, and their levels can be elevated in diabetes and restricted in animal models by certain antioxidant regimes (44).

Sell (582) used a C8 high-performance liquid chromatography (HPLC) column to measure Amadori product formation as the acid-hydrolyzed breakdown product furosine in the skin of rats and mice. Levels were found to increase at a rapid rate during aging of rodents. In the investigation of Sell et al. (590), the finding that furosine formation rates are better predictors of individual longevities in mice than those of CML, and pentosidine may best be explained by the biochemical origins of these products. Whereas furosine can originate only from glucose (591) and reflect cumulative glycemia (592), CML and pentosidine can originate from multiple sugars and are influenced by oxidative events that are catalyzed by transition metals and inhibited by antioxidative factors intrinsic and extrinsic to tissues (593). Furthermore, CML has multiple origins, including serine MPO (594) and LPO (595) reactions. In contrast, pentosidine is specific for the Maillard reaction (582).

The formation of the Amadori complex may be confirmed by isolation of its borohydride reduced product and by direct acid hydrolysis to furosine and pyridosine. Glucose and fructose are present in significant concentrations *in vivo* but react slowly in contrast to the ketoaldehydes and GOs, which react rapidly but are present at very low concentration *in vivo*. The rate of reaction of glucose with the lysines and arginines in a protein depends on the neighboring groups which influence the $pK_a$ of the side chain, for example, histidine (596).

Possible mechanisms have been reviewed, which include decreasing p*K* values, proximity to carboxyl groups, and the presence of phosphate. It is important to note that chelators, antioxidants, and sulfhydryl compounds have little effect on this initial stage of glycation. In the case of collagen, the specificity is unclear and has usually been based on particular cyanogen bromide (CB) peptides, the latter produced by cleavage of the collagen α-chain into well-established specific peptides. The results are equivocal, varying from attachment of ribose to all the CB peptides (597), glucose attachment to αICB6 (598), and preferential glycation of particular lysine residues in the short peptide α1CB3 and α2CB3,5 (599), although the latter constitutes almost two-thirds of the α2 chain. Wess et al. in 1993 (600) provided evidence from neutron scattering for glycation in the gap region of the fiber.

Both the Schiff base adduct and the keto-imine undergo further reactions with other amino acid residues (601) or follow metal ion-induced oxidative breakdown (602) to form AGEs. The term "AGE" is used to describe any protein-bound moiety detected after formation of the initial Schiff base-Amadori product. AGEs appear to be the final modification products. AGEs may result from a one-step conversion to $N^{\varepsilon}$-CML or involve a complex series of reactions to form an intermolecular cross-link such as pentosidine. In addition, oxidative breakdown can lead to the formation of more reactive sugars, such as 3-DG and the GOs (603), which then also complex with other lysines to form AGEs.

Besides the mostly known ones, several other compounds have been identified as glycation products. Two acid stable imidazolium compounds, designated as MOLD and GOLD, have recently been proposed as potential intermolecular cross-links, both of which are nonfluorescent. MOLD, a methylimidazolium compound, was originally proposed as a potential cross-link species by Brinkmann et al. (604) following its isolation from *in vitro* incubations of $N^{\alpha}$-hippuryllysine and MG. The major source of MG *in vivo* is from the nonoxidative degradation of triosephosphates, acetone, and ketone body metabolism and aminoacetone and threonine metabolism (605). The continual synthesis of MG *in vivo* could lead to a gradual buildup of imidazolium cross-linked collagen. Nagaraj et al. (606), isolated MOLD from *in vitro* glycated human serum albumin (HSA) and lens crystallin and showed a time-dependent increase, reaching levels of 7-8/HSA molecule and 5-6/α-crystallin subunit. Two mechanisms have now been proposed for MOLD formation. Both involve the reaction of two MG molecules and two lysine residues with a subsequent elimination of an acyl group, the first (604) prior to imidazolium formation, and an alternative proposed by Alabed et al. (607), in which hydrolytic deacetylation occurs after formation of a 2-acyl-4-imidazolium intermediate. The second imidazolium compound, GOLD (Fig. 1.23), has also been islolated from model *in vitro* systems following the incubation of $N^{\varepsilon}$-hippuryllysine and GO, a dicarbonyl formed by LPO and degradation of glucose and glycated protein (603).

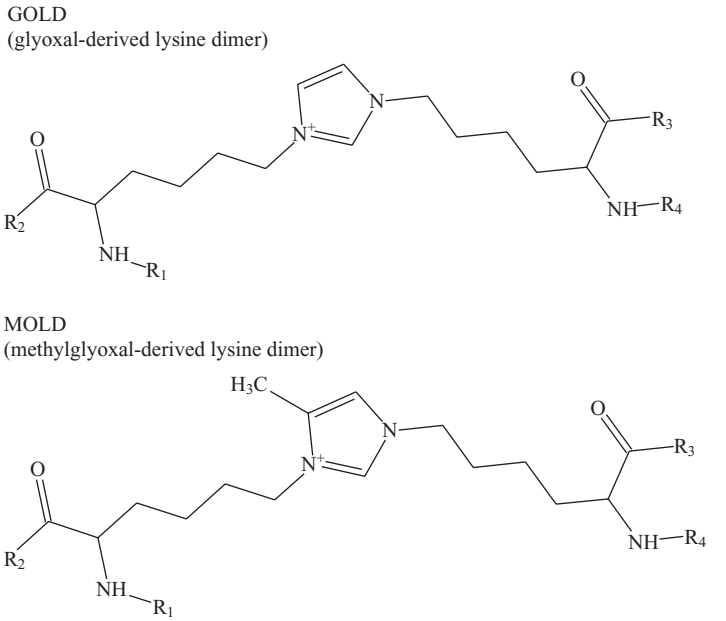



**FIG. 1.23** Structure of GOLD (glyoxal-derived lysine dimer) and MOLD (methylglyoxal-derived lysine dimer) (according to Ahmed, Biochem. J. 364: 1–14, 2002).

Three fluorescent cross-links, vesperlysines A, B, and C (Fig. 1.24), have recently been detected in the acid hydrolysates of *in vitro* glycated BSA (608). Incubation of protein in the presence of glucose results in the cross-linking of two lysine residues by a mixture of pyrrolopyridinium rings. The original six carbon skeleton of glucose is not incorporated into the cross-link structure, indicating that they are glyco-oxidation products derived from two molecules of glucose via dehydration, oxidative fragmentation, and condensation of the sugar moieties. In addition, they can be formed from a number of shorter chain sugars, for example, ribose and glyceraldehyde. Although peaks coeluting with authentic vesperlysines have been observed in the hydrolysate of glycated proteins, they have not been isolated from *in vivo* samples and therefore their presence *in vivo* remains uncertain.

Following *in vitro* reaction of glucose and $N^{\alpha}$-acetyl-lysine, Nakamura et al. (609) proposed crossline as a potential cross-link. Formed by the cyclocondensation of two glucose and two lysine molecules, crossline is a fluorophore existing in two epimeric forms, A and B (Fig. 1.25). In contrast to candidates described previously, they are acid labile and have not been isolated from tissues. However, immunohistochemical studies using sera derived from $N^{\alpha}$-acetyl derivatives has indicated their presence in both *in vivo* and *in vitro* glycated protein.

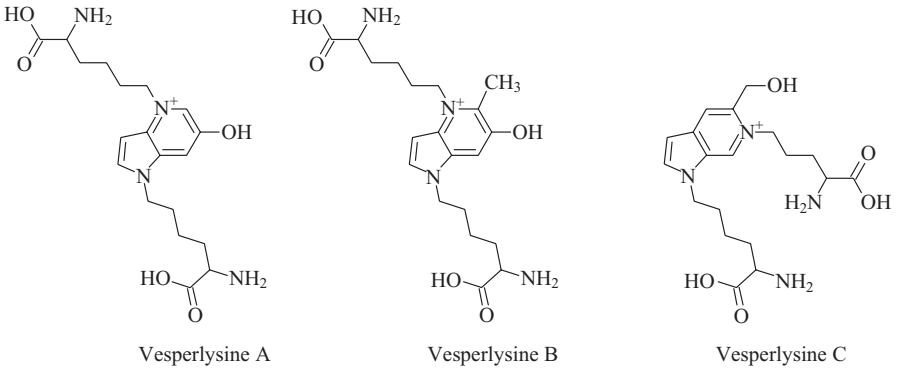



**FIG. 1.24** The three different isoforms (A, B, and C) of vesperlysine, a fluorescent cross-link between two lysine residues resulting from glycation (according to Paul, Biochem. J. 330: 1241–1248, 1998).

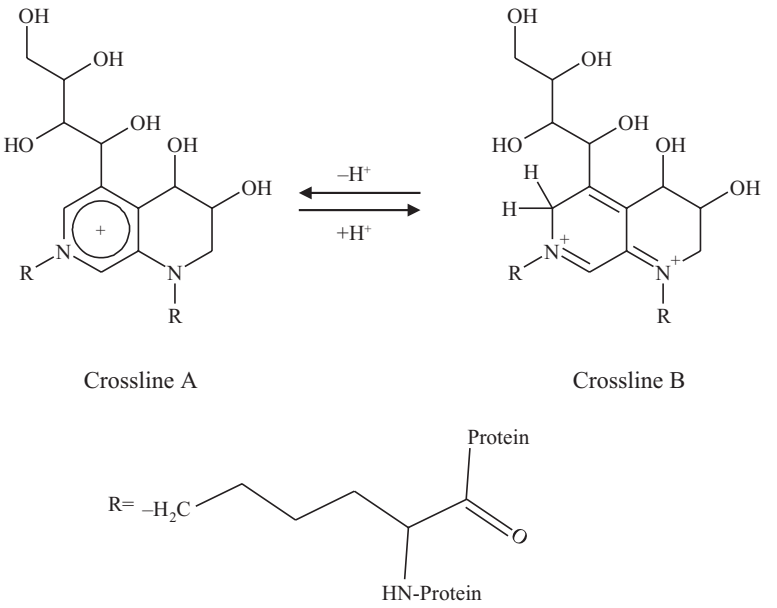
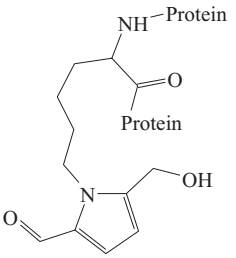



**FIG. 1.25** The potential cross-link crossline is formed by the cyclocondensation of two glucose and two lysine molecules. The fluorophore crossline exists in two epimeric forms A (left structure) and B (right structure) (according to Bailey et al., Mech. Ageing Dev. 106: 1–56, 1998).




Pyrraline

**FIG. 1.26** Chemical structure of pyrraline, a reaction product of a lysine residue and 3-DG (according to Bailey et al., Mech. Ageing Dev. 106: 1–56, 1998).

Pyrraline (Fig. 1.26) is another lysine derivative, and has also been identified in a variety of tissues by the use of antibodies (610) and by chromatographic techniques (611). The mechanism of formation is suggested to involve 3-DG as the immediate precursor and reaction with the o-amino of lysine to form 5-hydroxymethyl-1-alkyl pyrrole-2-carbaldehyde (612).

Argpyrimidine, a pyrimidinium compound, has been shown to be formed during the *in vitro* incubation of MG and $NH_2$. Two mechanisms for its synthesis have been proposed: one via the formation of double Schiff base adducts (607) and the second through a 3-hydroxypentane-2,4-dione intermediate (613). It is acid labile and has distinctive fluorescent characteristics which resemble that of modified protein. However, it has not been isolated and definitively characterized from an *in vivo* glycated protein.

Glycation of the α-amino terminal group occurs, but to a lesser extent than the greater availability of the numerous lysine and arginine side chains. However, following reaction with GO, Krause et al. (614) characterized a new class of AGEs as pyrazinones (Fig. 1.27).

The importance of AGEs has been demonstrated by the administration of AGEs (prepared *in vitro* by glycation of serum albumin) to normal rats, which revealed typical age- and diabetes-related changes, for example, basement membrane thickening, glomerula hypertrophy, and increase in mesangial volume in the total absence of hyperglycemia (615). In this context, diet may be a significant source of AGEs. Among commonly consumed foods, fat showed the highest levels of AGEs, meat about half this value and carbohydrate the lowest. The actual values obviously increase with the cooking temperature (616, 617).

CML, CEL, GOLD, MOLD, and the fluorescent cross-link pentosidine are known to increase in lens protein and tissue collagen with age (618–620). 3-Deoxyglucosone may be formed nonenzymatically (621). MG is formed by both enzymatic pathways (from threonine and acetone) and by nonenzymatic pathways (622). Nitric oxide blocks the antiproliferative effect in VSMCs

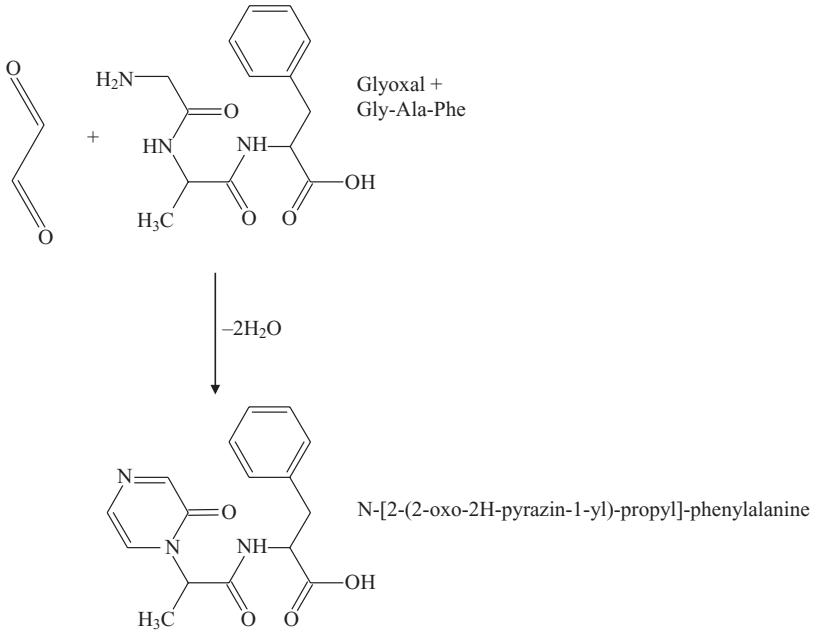
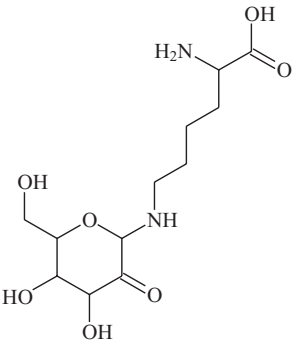



**FIG. 1.27** Reaction of GO with a short peptide containing glycine, alanine, and phenylalanine, resulting in the formation of a pyrazinone, a class of AGEs (according to Krause et al., Amino Acids 27: 9–18, 2004).

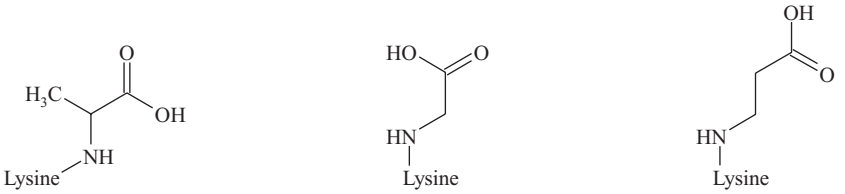
(623). The vasodilatory response to nitric oxide correlates with the level of AGEs (624).

The major product of glycation in tissue proteins is fructoselysine (FL) (Fig. 1.28), formed by glycation of ε-amino groups on lysine residues. The extent of glycation of tissue proteins depends on the ambient glucose concentration and is relatively constant with age. However, products formed from FL in subsequent Maillard or browning reactions accumulate gradually with advancing age in long-lived tissue proteins (625, 626). These products include $N^{\varepsilon}$-CML and $N^{\varepsilon}$-(carboxymethyl)hydroxylysine (CMhL), which are formed by oxidative cleavage of FL and glycated hydroxylysine, respectively, pentosidine, a fluorescent cross-link formed between arginine and lysine residues (627–628), as well as unidentified compounds with characteristic Maillard-type, visible wavelength fluorescence (maxima at $\lambda_{ex}$ = 328 nm, $\lambda_{em}$ = 378 nm). CML, CMhL, and pentosidine have been termed "glycoxidation" products (629) because both glycation and free-radical oxidation reactions are required for their formation from reducing sugars (Fig. 1.29). The exact origin of these compounds, that is, from glucose versus ribose (630), ascorbate (627, 628), or other sugars (629), is uncertain, although present evidence suggests that they are derived exclusively from reactions between proteins and carbohydrates (631).




Fructoselysine

**FIG. 1.28** The formula of FL, that can be formed by glycation of ε-amino groups on lysine residues.




CEL (Nε-Carboxyethyl-lysine )

CML (Nε-Carboxymethyl-lysine )

Lactatolysine

**FIG. 1.29** The chemical structures of CEL (Nε-carboxyethyl-lysine), CML (Nε-carboxymethyl-lysine), and lactatolysine, formed by the oxidative degradation of the Amadori product via an intermediate after the reaction of GO with lysine (according to Bailey et al., Mech. Ageing Dev. 106: 1–56, 1998).

According to the "glycation hypothesis" of aging and the pathogenesis of diabetic complications, accumulation of end-stage products of the Maillard reaction (also known AGEs) alters the structural properties of tissue proteins and reduces their susceptibility to catabolism (632). These changes contribute to the aging of tissues and, when accelerated by hyperglycemia, to the gradual development of diabetic complications.

***1.3.3.1 Receptor for Advanced Glycation End Products (RAGE)*** A number of AGE-specific receptors have now been described, although Shaw and Crabbe (633) have suggested that the interaction of AGEs and macrophages is predominantly nonspecific. The interactions between AGE and receptors have also been linked to the development of pathological conditions

resulting from AGE formation. Interaction with RAGE (receptor for advanced glycation end products) is thought to play a central role in the onset of vascular disease in diabetes through chemotaxis, generation of cellular oxidant stress, and associated increase in the levels of vascular cell adhesion molecule-1 (VCAM-1). This has the effect of increasing attachment of mononuclear macrophages to the vessel wall and results in the generation of a pro-inflammatory environment necessary to promote vascular pathology (634). A cascade of events that is thought to be linked to the activation of NFκB sites within the promoter of VCAM-1 is instigated by the binding of AGEs to RAGE and increased oxidative stress (635).

***1.3.3.2 $N^{\varepsilon}$-Carboxymethyllysine and $N^{\varepsilon}$-Carboxyethyllysine*** $N^{\varepsilon}$-CML is a nonfluorescent product (602) which has been shown to accumulate with age (626, 631). Several mechanisms of formation have been reported: oxidative degradation of the Amadori product (602, 626), the reaction of GO with lysine (636), and by oxidation of both the Schiff base and the Amadori product (637). $N^{\varepsilon}$-(Lactatolysine) (LL) is also formed by the oxidative degradation of the Amadori product via a 3,4-ene-diol intermediate. Formed during the degradation of synthetic fructosamine, it has also been detected in proteins glycated *in vitro*, for example, in lens protein (638). $N^{\varepsilon}$-CEL, a homologue of CML, has also been detected in lens protein at levels comparable to CML, and has been shown to increase with age and has been proposed as a marker of oxidative stress. It can be formed *in vitro* by the reaction of lysine or protein with a number of carbonyl compounds, the highest yields being with MG and triose phosphates (620).

The CML content of proteins is increased in actinic dermal aging (639) and has been correlated with diabetic complications (640). Furthermore, CML accumulates in skin lesions of actinic elastosis, a hyperplasia of the upper dermis caused by chronic exposure to sunlight (639, 641). The CML content of proteins can be used as a biomarker for intracellular glyoxidative stress (637). It has been shown that CML serves as a chelating agent that promotes MCO, leading to carbonyl formation at specific sites in a protein, thereby increasing the oxidative damage to the protein (642).

***1.3.3.3 Pentosidine*** Pentosidine is an imidazo pyridinium compound (630) derived from lysine, arginine, and pentose. It has been identified as the end product of the Maillard reaction elicited by hexoses, pentoses, ascorbate, and a variety of Amadori compounds *in vitro* (627, 631). Moreover, it has been identified in numerous collagenous and noncollagenous tissues and shown to exhibit a linear increase with age (630).

The importance of sugars, especially glucose, as a possible mediator of the aging process was hypothesized by Cerami (580). In essence, the open-chain configuration of the sugar forms a Schiff base with a free amino group on the protein, which in turn can rearrange to form a more stable Amadori product. Susceptibility to nonenzymatic glycosylation, or glycation, is highly dependent

on the concentration of the protein and its microenvironment (i.e., accessibility of its free amino groups). The Amadori product, in turn, can undergo a complex series of reactions leading to the Brown products and cross-links referred to as Maillard products or advanced glycosylation end products (580). Pentosidine was discovered and isolated from old human dura mater (630). Since its mechanistic formation involves both glycation and oxidation, the term "glycoxidation product" was coined by Baynes (629) to delineate a novel class of Maillard products resulting from oxidative cleavage of Amadori products. Baynes (629) concluded that pentosidine could serve as a biomarker for carbohydrate-dependent damage to protein and an indicator of the extent of underlying chemical modification, oxidation, and cross-linking of tissue protein caused by reducing sugars. Levels of pentosidine have been shown to progressively increase with age in various tissues of human origin, including dura mater, skin, and cartilage. Although originally isolated from the highly insoluble fraction of collagen-enriched dura mater (630), pentosidine has also been identified in various other tissues and proteins (643). A factor that may be involved in controlling glycoxidation rates is glycemia. Cerami (580) hypothesized that glucose served as a mediator of aging on the basis of findings that complications of aging such as increased stiffening of joints, arteries, lung, and heart; bone loss; loss of lens accommodation; cataract formation; atherosclerosis; and cardiovascular disease are dramatically worsened by diabetes. Indeed, these age-related changes, as well as glycoxidation rates, occur twice faster in diabetes (644), and diabetes thus resembles accelerated aging.

### 1.3.4 Racemization

Some amino acid residues spontaneously racemize from the L-configuration to the D-form. D-Serine, D-threonine, D-aspartic acid, and D-tyrosine have been detected in long-lived proteins such as eye lens proteins and enamel and dentine of teeth (645). These changes may play causative roles in pathology as D-amino acids appear enriched in lenticular cataracts and in the amyloid peptide associated with AD, but the occurrence of these changes may merely reflect the long-lived nature of the aberrant protein accumulations (59). L-Isoaspartyl residue and racemized D-aspartyl residue can be recognized and repaired by a methyltransferase (646).

The isomerization and racemization of aspartyl residues is a well-known aging effect in long-lived proteins (647), and has recently been shown to occur more in the C-telopeptides (648) and in the N-telopeptides (649) of type I collagen.

### 1.3.5 Nitrosylation

Nitrosylation, which is a highly conserved posttranslational mechanism, is recognized to regulate the function of a spectrum of proteins (288). Nitrosylation, the covalent attachment of a nitrogen monoxide group to the thiol side

chain of Cys, depends on the redox milieu in that region of the protein. The ratio of superoxide/$^{\bullet}$NO production by NOS is an important determinant of the redox milieu. It is established that both skeletal (650) and cardiac (651) RYRs are, in fact, activated by S-nitrosylation (652). The cardiac ryanodine isoform, which is S-nitrosylated under basal conditions, has been shown to colocalize with NOS1 in the SR (653).

Nitric oxide signaling involving protein posttranslational modifications became relevant for mitochondrial physiology as an *in vivo* phenomenon after that proteomic analysis showed that rat aging is associated with an almost specific nitration of the $Tyr^{269}$ of the β-subunit of F1-ATPase. Mitochondrial proteins such as ATP synthase, creatine kinase, and aconitase are inhibited by nitration or $ONOO^{-}$-mediated oxidation of cysteinyl residues (654).

### 1.3.6 Tyrosyl Radicals and Nitrotyrosines

Whereas most amino acids can be oxidized, ROS-induced oxidation of, in particular, tyrosine and Cys may have profound effects on cell function, as these residues are often located in the active site of enzymes. Oxidation of essential tyrosine residues has been observed for SOD (655) and GSH reductase (GR) (656), resulting in their inactivation. Intermolecular cross-linking of enzymes involved in signal transduction by dityrosine formation has been detected in cells (657). Tyrosine oxidation may lead to the formation of tyrosyl radicals. These radicals are formed by reaction with hypochlorite, peroxynitrite, or by radicals formed in transition metal ion-catalyzed Fenton and Haber–Weiss reactions (e.g., $H_2O_2/Fe^{2+}$) (658). Peroxidases are also important sources of tyrosyl radicals. Protein tyrosyl radicals may form intra- or intermolecular o,o′-dityrosine bonds. It is known that the concentrations of o,o′-dityrosine bonds increase with aging in heart, skeletal muscle, and lens proteins (659). Another oxidation product is 3-NY that can be introduced into proteins as a result of tyrosine nitration by peroxynitrite, a product of superoxide ($O_2^{\bullet-}$) and nitric oxide ($^{\bullet}$NO) (660), by peroxidase-catalyzed oxidation of nitrite (146) and by nitric oxide reaction with protein tyrosyl radicals (661). MPO-driven oxidation systems produce 3-chlorotyrosine (662), which interestingly can be detected in the human atherosclerotic intima (194).

Peroxynitrite, formed as a result of superoxide and nitric oxide interaction, is able to attack Tyr either by a pathway via homolytic hydroxyl radical-like reaction, resulting in protein-based carbonyls, or via an electrophilic nitration (663–664). In the presence of bicarbonate, peroxynitrite forms a $CO_2$ adduct, which augments its reactivity. Formation of 3-NY by this route has become the classical protein marker specifically for the presence of peroxynitrite (665).

Pathologic and aging tissue often shows increased levels of 3-NY (666). In addition, 3-NY levels may serve as an indicator for the progression of cardiovascular disease and its modulation by statin therapy (667). In general, the formation of 3-NY is taken as evidence that tissue is exposed to some sort of

oxidative (or nitrative) stress. *In vivo*, the skeletal muscle creatine kinase suffers nitration of both $Tyr^{14}$ and $Tyr^{20}$, but not measurably of any of the other seven Tyr residues at positions 39, 82, 125, 140, 173, 174, and 279. Tryptic peptides containing all these remaining Tyr residues were covered by MS/MS analysis of the protein, indicating the validity of the analytical method. In contrast, the *in vitro* exposure of creatine kinase to peroxynitrite in the presence of $CO_2$ resulted in the exclusive nitration of $Tyr^{82}$ (668). Again, tryptic peptides containing all of the other eight Tyr residues were covered by MS/MS analysis. Nitration of tyrosine residues within the GSH disulfide binding site of GR resulted in a nearly 1000-fold decrease in catalytic efficiency of the enzyme (669).

It is well established that structural analogues of L-tyrosine, such as 3-iodotyrosine, 3-fluorotyrosine, and 3,4-dihydroxy-L-phenylalanine, are incorporated by posttranscriptional mechanisms into α-tubulin (670). The mechanism appears to involve tubulin L-tyrosine ligase, an enzyme that seems to have promiscuous substrate specificity (671, 672). 3-NY generated by RNS can subsequently be incorporated into tubulin by this mechanism (671).

Modification of tyrosine residues in receptor molecules has been shown to impair signaling pathways (673); for example, nitration is able to block the cycle of phosphorylation/dephosphorylation of tyrosine (674). In fact, nitration of tyrosine blocks its phosphorylation (675, 676). This was demonstrated for the insulin receptor containing key tyrosine residue sensitive to nitration (677).

Therefore, oxidative damage to key signaling proteins might directly contribute to age- or disease-related changes of cellular metabolism (665, 678, 679) Ischiropoulos et al. established that a nitrated tyrosine hydroxylase is selectively degraded by chymotrypsin and the proteasome (680). Interestingly, the existence of a repair enzyme for nitrated tyrosines, the "nitrotyrosine nitrase," was also proposed, but not finally shown (681).

Leeuwenburgh et al. (682) demonstrated an increase of 3-NY levels with age in rat and mice liver. However, the results suggest that proteins oxidized by RNS do not accumulate dramatically. Therefore, an investigation by Viner et al. on the SERCA2a nitration from rat skeletal muscle demonstrated an increase with age, but only one out of four Tyr were modified (683). However, further studies suggest that this might already reduce SERCA2a function (684). 3-NY has also been reported to be increased in neurodegenerative diseases such as AD, PD, and HD (685, 686).

***1.3.6.1 Dityrosines*** Dityrosine cross-links, which apparently arises following the reaction between two tyrosyl radicals, are generated by peroxidases and other heme proteins. Dityrosine cross-linking of proteins has been found to increase with age in mouse skeletal muscle and heart, but not in the brain or liver (27).

Heinecke et al. reported that o,o′-dityrosine generated by activated phagocyte causes cross-links in proteins and lipoproteins *in vitro*. o,o′-Dityrosine

levels in tissue are elevated in atherosclerosis, inflammatory lung disease, neurodegenerative disorders, and aging (313, 687). Kato et al. developed a monoclonal antibody for detecting protein dityrosine, and the antibody reacted with peptidyl dityrosine, derived from Thr-Thr-Ser, rather than free dityrosine (688).

Tyrosine oxidation products do not appear to be as abundant in biological samples as protein carbonyls. Dityrosine levels may be even lower (44). This relatively low degree of modification may result from the lower presence of Tyr in proteins compared to the sum of the several amino acids that form carbonyls (Lys, Arg, Pro, Thr) (689).

### 1.3.7 Protein Carbonyls

Attacks of ROS on proteins have been shown to increase their carbonyl content due to the formation of aldehydes and ketones in certain amino acid residues (690). Much of the evidence for accumulation of oxidative damage with age comes from an increase in the protein carbonyl content of tissues. The generation of carbonyl derivatives is orders of magnitude greater than other kinds of protein oxidation, and the carbonyl content of proteins has become the most generally used method for estimation of oxidative stress-mediated protein oxidation.

Direct oxidation of lysine, arginine, proline, and threonine residues may produce carbonyl derivatives. In addition, carbonyl groups may be introduced into proteins by reactions with bifunctional aldehydes such as 4-hydroxy-2-nonenal or MDA produced during LPO (690). Still another route of protein carbonylation is through the oxidation of sugars that nonenzymatically derivatize lysine in the glycation process (691). Protein carbonyls were shown to exhibit age-associated increase in the mitochondria (384). The findings from Agarwal and Sohal (385) demonstrated that proteins in the mitochondria of 15-day-old flies had 65% more carbonyls in total than those from 5-day-old flies. The intracellular levels of protein carbonyls were shown to increase in aged human dermal fibroblasts (31) and in human brain (692), human eye lens (693), and human plasma (694).

Protein carbonyl derivatives are shown to be formed by several chemical mechanisms, such as the cleavage of the polypeptide chain by the α-amidation and glutamic oxidation pathways (3), by the Michael addition of amino acid side chains (histidine imidazole groups, lysine amino groups, and Cys sulfhydryl groups) to α-β-unsaturated aldehydes, by reactions of lysine amino groups with a bifunctional aldehyde as MDA, or as a result of glycation/glycoxidation reactions. Some of the formed products, as CML, are reasonably strong metal ion chelators and are able to promote further oxidation. In view of the fact that protein carbonyl groups are generated by several different mechanisms, it is not surprising that the concentration of protein carbonyl groups is orders of magnitude greater than any other kind of protein oxidation (3).

### 1.3.8 Aldehyde–Protein Reactions

Lipid molecules are particularly vulnerable to oxidative attack because their unstable reactive double bonds can set off a series of peroxidative chain reactions (695). Among the products are reactive aldehydes such as MDA, acrolein, GO, and HNE, all of which can react with protein amino acid side chains, especially those of lysine, arginine, and histidine. Many of the reaction products, called advanced LPO end products or ALEs, possess carbonyl functions which lend themselves to cross-linking to either unmodified polypeptide chains or altered proteins (581).

4-HNE is known to inflict cell damage, redox disturbance (695), and various other deleterious processes (696). 4-hydroxyhexenal (HHE) is a reactive byproduct of *n*-3 fatty acid peroxidation (695) and is structurally similar to HNE, which is derived from *n*-6 fatty acids, but its biological actions and efficacies may vary greatly from HHE. LPO breakdown products such as HNE, MDA, and acrolein bind covalently to Lys, His, and Cys residues, leading to the addition of aldehyde moieties to the protein (697). The binding of lipid reactive products to proteins is a common occurrence, causing vascular damage under oxidative stress (698). In membranes, competition and interactions between protein and lipid oxidation are expected. The fluorescent cross-links that can form between lipid oxidation products and proteins possibly contribute to ceroid, lipofuscin, and other "age pigments" found in cells (44).

Radicals produced during lipid autoxidation can inactivate $\alpha_1$-proteinase inhibitor by oxidation of Met-358 (699). Later studies with liver homogenates also demonstrated a slow inactivation of GPx (700). End products of lipid oxidation such as MDA and HNE (701) are also inactivating agents, possibly via Schiff base formation. Schiff bases are short-lived species formed by the reaction of carbonyl groups with amines, and can be formed, for example, during exposure of proteins to lipid-derived aldehydes (702), autoxidizing sugars, and amino acid-derived aldehydes. Thus, binding of the apolipoprotein B (apoB) protein of LDL to its cellular receptor is perturbed by reaction with HNE at modest levels (703), and grossly altered when aggregating, supra-pathological levels are used. With some lipid carbonyls, Schiff base formation may be of limited importance compared with Michael addition reactions (704). In insulin (which lacks free thiol groups), histidine residues are selectively modified by Michael addition of HNE (705). With dialdehydes, Schiff base formation and Michael addition might occur simultaneously, thus cross-linking lysine residues (706). HNE also inactivates G6PDH and GAPDH; in the latter case, both intra- and intermolecular cross-links appear to be formed (707). Carbonic anhydrase III contains several Cys, histidine, and lysine residues that make it a target of HNE (708).

In a study by Liu et al (397) MDA was more potent than HNE in affecting carnitine acetyltransferase (CarT) kinetics. This may be because 4-hydroxyalkenals are highly specific reagents for SH groups, although they may also modify lysine, histidine, serine, and tyrosine, and MDA can readily modify

proteins under physiological conditions, although it is less reactive with free amino acids. MDA reacts primarily with lysine residues and can then form more stable intra- and intermolecular cross-links (695).

***1.3.8.1 MDA-Protein Adducts*** MDA is able to produce fluorescent cross-links with proteins. The formation of 1,4-dihydropyridine-3,5-dicarbaldehydes are the most likely produced fluorophores under physiological conditions (709). Recent studies show that levels of $N^{\varepsilon}$-(malondialdehyde)lysine (MDAL) in proteins increase during aging in rat tissue (710) and that elevating the MDAL content alters protein properties including increased aggregation, sensitivity to degradation, and secondary ROS generation (711).

***1.3.8.2 4-Hydroxy-2,3-Nonenal-Protein Adducts*** The cytotoxicity of LPO is attributed partially to its ability to modify proteins. Aldehydes and especially 4-HNE is one of the most reactive and abundant products formed by the oxidation of ω-6 PUFAs (695). Therefore, high HNE levels were detected in a number of pathological situations and diseases (712–714). The steady-state HNE levels are below 1 μM, but these are able to increase to about 10 μM under pathophysiological conditions (695). Since HNE is highly reactive toward proteins, it modifies a large array of polypeptides (707). To prevent the HNE-mediated protein modification, several HNE-metabolizing enzymes have evolved, including GSH S-transferase, aldehyde dehydrogenase, and aldose reductase (695).

HNE-protein adducts have been found in several age-related studies. HNE modification directly correlated with loss of resistance of actin to detergent extraction, suggesting cytoskeletal damage in aging RPE, contributing perhaps to age-related macular degeneration (715). Proteomics studies have reported a large number of proteins in AD brain that showed increased levels of protein-bound HNE, including: ATP synthase, α-enolase, aconitase, aldolase, GS, MnSOD, peroxiredoxin 6, dihydropyriminidase-related protein 2 (DRP-2), and α-tubulin (716).

### 1.3.9 Cross-Linking of Proteins

The cross-linking theory of aging has a long history. Bjorksten (717) proposed an aging theory based on the loss of function of all proteins by excessive cross-linking through the reaction with aldehyde metabolites. Michael addition of either a histidine, lysine, or Cys residue of a protein with an α-β-unsaturated aldehyde, such as HNE, leads to the formation of an aldehydic adduct that can react with the lysine residue of another protein to form a Schiff base protein–protein cross-linkage. Protein–protein cross-links can also be formed by MDA (29).

Protein oxidation always generates a certain amount of inter- and intra-protein cross-links, often by a multitude of mechanisms, including a Schiffs

base formation of a protein carbonyl group and a lysine, the pairing of various carbon-centered radicals, Michael additions to double bounds, and -S-S- cross-links (8). Thus, cross-linked proteins are not only resistant to proteolytic degradation but they are also inhibitors of the protasomal pathways (718) (see Chapters 2 and 3).

## 1.4 THE OXIDATION OF EXTRACELLULAR MATRIX, MEMBRANE AND CYTOSKELETAL PROTEINS

It was reported that ECM generated by mesangial cells is susceptible to MCO and that this alters its adhesive properties (263, 719). A characteristic feature of glomerular disease is an accumulation of ECM, creating a disorganized and perhaps biochemically modified array of proteins that may result in an environment inhospitable to normal mesangial cell growth and function. ECM accumulation is the net result of a balance between ECM generation and ECM degradation (720). In advanced glomerular disease, particularly when active inflammatory stimuli have often ceased, it is unclear why the mesangial ECM should remain in excess.

Given the balance between generation and degradation, it is plausible that normal mesangial clearance mechanisms may be unable to degrade this excess ECM due to modifications in the ECM, making it resistant to these enzymes. Glomerulosclerosis is characterized by an accumulation of ECM proteins and a paucity of glomerular mesangial cells and can be seen as an end result of glomerular injury and in aging. ECM may become oxidized as a part of inflammatory renal injury and with aging. Mattana et al. (721) evaluated the hypothesis that oxidation of mesangial ECM could alter its susceptibility to the action of ECM degrading enzymes. Radiolabeled mesangial ECM was generated by growing cells on tissue culture plastic and incubating with [$^3$H]proline. After removal of cells, leaving behind ECM, selected wells were oxidized using a $FeCl_3$/EDTA/ascorbate system or treated under control conditions. The control and oxidized matrices were then incubated with concentrated supernatants from mesangial cells containing the major mesangial ECM degrading enzyme, the matrix metalloproteinase-2, whose activity was confirmed by gelatin substrate zymography. Counts released corresponding with ECM degradation were measured. ECM oxidized with this system was significantly less susceptible to degradation compared with control ECM. To confirm that this effect was specifically due to oxidative modification of the ECM rather than changes unrelated to oxidation, ECM was coincubated with the oxidizing system plus the radical spin trap N-tert-butyl-α-phenylnitrone (PBN). PBN treatment was able to prevent the impaired susceptibility to degradation induced by exposure to the oxidizing system. Exposure of ECM to milder oxidative stress, however, modestly enhanced susceptibility to degradation. These data suggest that oxidation of mesangial ECM can modulate its susceptibility to degradation. This

may account for the development of ECM accumulation and glomerulosclerosis in inflammatory renal injury and in aging.

Recent investigations show that glycosaminoglycans (GAGs) and proteoglycans have the ability to affect LPO (722). A protective effect of these ECM components has been demonstrated in various experimental systems, including fatty acids and liposomes, where oxidation was induced by transition metals, including copper and iron. The effect was specific and dependent on the type and structural features of GAGs and proteolycans. The mechanism of peroxidation inhibition was likely to be dependent, at least to a large extent, on the sequestration of transition metals by GAG chains. Thus, it is conceivable that GAGs in the ECM and in the pericellular space may contribute to protecting cells against free-radical damage. It is of particular interest that in certain tissues (cornea and aorta), aging was associated with a decrease of content of the GAGs, which were most effective as antioxidants. This suggests that age-induced modifications of ECM composition in certain tissues may increase the susceptibility to oxidative stress.

### 1.4.1 Collagen

The majority of the collagens can be classified according to the nature of their aggregated forms: the fibrous collagens, collagenes type I, II, III, and the minor collagens V and XI. The network collagens are the type IV molecules. Filamentous collagen is the type VI. The fibril-associated collagens are type IX, types XII and XIV. Many of the remaining collagen types are known only by their DNA sequence, and as such their macromolecular structures are unknown. Bone and tendon are predominantly fibrous type I collagen, the vascular system contains both types I and III, while cartilage contains predominantly type II collagen. The thin basement membranes are primarily type IV collagen. Changes in the collagenous matrix are readily observed during development (723) and are often reported as age related, mainly because the differences in old age are harder to identify. However, it is now becoming clear that there are significant quantitative and qualitative changes in the collagenous tissues in old age. However, one study has indicated that in the last few decades of life there is an increase in the proportion of type III collagen present in the dermis (724). This effect could be due to a loss or reduced synthesis of type I collagen or an increase in type III collagen due to a change in the phenotypic expression of the fibroblasts in old tissue.

Recently, type I collagen has been shown to undergo β-isomerization of Asp-Gly within the C-telopeptide (648). This modification was demonstrated in bone tissue by direct bone analysis and indirectly in urine, and the extent of isomerization was shown to increase with age. The overall shape and function of the human skeletal system, in terms of flexibility and locomotion, depend on a basic framework of collagen fibers (725). The collagen fibers are essentially inextensible and therefore provide mechanical strength, and through that strength confer and maintain form while allowing flexibility

between various organs of the body. Thus, the randomly oriented fibers of the skin permit considerable extension of the tissue. The fibers of tendons are aligned in parallel and therefore loaded instantly, permitting maximum transfer of the energy of muscle contraction to the skeleton. The fibers of bone are organized in concentric layers to maximize for torsional and compressive stresses and the rigidity is conferred on the bone by mineralization. The surface of cartilage on the bone extremities allows efficient movement of the skeleton through lubricating joints following muscular contraction, and stability is provided by a small proportion of fine collagen fibers in a mainly polysaccharide gel. The cornea provides an example of well-ordered fibers in precise layers at a defined angle to each other, thereby allowing the transmission of light. In contrast to these fibrous structures, the network structure of basement membranes provides a filtration system and an attachment site for cells, for example, the kidney glomeruli and arterial basement membranes. This biological diversity of function of collagenous tissues is primarily due to the fact that these fibers are biopolymers of one of several genetically distinct collagens, which are to some extent tissue specific (725).

Young collagen fibers are tough and in bulk appear white. At the same time, they are easily pliable and can be solubilized by concentrated urea or by enzymatic digestion. With age, however, they normally undergo progressive changes characterized by decreased solubility and elasticity, increased resistance to enzymatic digestion, and the accumulation of fluorescent and yellow pigments. The nature of these physical and chemical changes suggests progressive cross-linking. Among several hypotheses that have been proposed for the origin of cross-links of the ECM, the possibility that some of these are sugar derived is based on the fact that reducing sugars, when incubated with proteins, can form fluorescent and yellow-colored adducts and cross-links resembling those occurring in normal aging and hyperglycemia as in diabetes (726).

During aging, changes occur in the collagenous framework. These changes in the physical properties of the fibers are reflected in the well-documented increases in stiffness of skin, tendon, bone, and joints in old age. The major changes are an increase in rigidity of the tissue, the fibers ultimately becoming brittle (727). Such changes are clearly deleterious to the optimal functioning of the locomotive system, the elastic vascular system, and the filtration properties of the basement membranes.

To investigate the contribution of glycation and oxidation reactions to the modification of insoluble collagen in aging and diabetes, Maillard reaction products were measured in skin collagen from 39 patients with type 1 diabetes and 52 control subjects without diabetes (631). Compounds studied included FL, the initial glycation product, and the glycoxidation products, $N^{\varepsilon}$-CML and pentosidine, formed during later Maillard reactions. Collagen-linked fluorescence was also studied. In nondiabetic subjects, glycation of collagen (FL content) increased only 33% between 20 and 85 years of age. In contrast, CML, pentosidine, and fluorescence increased fivefold, correlating strongly with age. In diabetic patients, collagen FL was increased threefold compared with

subjects without diabetes, correlating strongly with glycated hemoglobin but not with age. Collagen CML, pentosidine, and fluorescence were increased up to twofold in patients with diabetes compared with control patients; this could be explained by the increase in glycation alone, without invoking increased oxidative stress. There were strong correlations among CML, pentosidine, and fluorescence in both groups, providing evidence for age-dependent chemical modification of collagen via the Maillard reaction, and acceleration of this process in diabetes. These results support the description of diabetes as a disease characterized by accelerated chemical aging of long-lived tissue proteins. Dunn et al. (626) showed some increase in skin collagen with age. Human studies aimed at the assessment of glycation in collagen originally had shown increases in skin, tendon, and glomerular basement membrane over a life span (728, 729). Collagen glycation either does not vary or increases modestly with age. Sell (582) measured Amadori product formation as the acid-hydrolyzed breakdown product furosine in the skin of rats and mice. Levels were found to increase at a rapid rate during aging of rodents. Possible reasons for the age-related increase in glycation in rodent skin collagen are age-related declines in glucose tolerance and collagen turnover.

The degeneration of intervertebral disks is frequently associated with a unique browning of the tissue, which mostly results from nonenzymatic glycosylation of collagen (730). Nonenzymatic glycosylation of collagen involves the attachment of sugar by reactive oxygen and a series of rearrangement and dehydration steps (731, 732). Yang et al. (733) described the preparation and application of a structural conformation-dependent antibody specific for an abnormal epitope on the aged collagen II from annulus fibrosus. Collagen molecules can be posttranslationally modified either by enzymatic or nonenzymatic processes. Collagen II was isolated from annulus fibrosus of five donors with ages from 6 up to 83 years. Purified collagen II was subjected to the analysis. Both hydroxylation and glycosylation of collagen II did not show a significant change during aging. During the extraction, it was found that the solubility of tissue by limited pepsin digestion decreased greatly with age. This mostly results from the intermolecular cross-linking by nonenzymatic glycosylation and by attachment of lipid peroxides. Accordingly, collagen II extracted from aged annulus fibrosus shows a higher relative fluorescence, which is characteristic for nonenzymatic glycosylation and attachment of lipid peroxide to protein.

Physiological cross-linking of collagen in growth and maturation is initiated by the enzyme lysyl oxidase in the presence of $Cu^{2+}$ and the quinone cofactors. This enzyme oxidizes the ε-amino group of a lysine residue to an aldehyde which acts only upon telopeptide residues when bound to a highly conserved amino acid sequence (Hyl-Gly-His-Arg) opposite the N- and C-terminals of an adjacent quarter-staggered aligned molecule. This forms the collagen cross-linking precursor α-aminoadipic acid-δ-semialdehdye, also known as allysine (734, 735). Allysine can also be formed during MCO, as originally reported by Stadtman (29) and more recently by Suyama and associates (736), who have

proposed a mechanism for allysine formation based upon MCO reactions of α-dicarbonyl sugars with lysyl residues. Allysine itself probably undergoes a further oxidative reaction, leading to the formation of 2-aminoadipic acid *in vivo* (737). 2-Aminoadipic acid, but not 6-hydroxynorleucine, significantly and progressively increased with age to very large levels and was significantly elevated by diabetes and renal failure. The finding that 6-hydroxynorleucine was the foremost marker related to 2-aminoadipic acid levels in the study of Sell et al. (738) provides further proof that allysine is a precursor for 2-aminoadipic acid in human skin collagen. Second, since both CEL and CML were also significantly correlated with 2-aminoadipic acid levels, it was suggested that the oxidative mechanism for 2-aminoadipic acid formation is related to formation of these products *in vivo*. Inhibition of the enzyme lysyl oxidase during copper deficiency and the disease lathyrism has long been known to have profound effects on all collagenous tissues, leading to extreme fragility due to the subsequent reduction in cross-linking. The activity of lysyl oxidase has been found to decrease with age, although the rate depends on the tissue (739).

The telopeptide lysine aldehydes condense with either lysine or hydroxylysine residues in the conserved sequence of the triple helix to form reducible Schiff bases. In tissues such as skin and rat tail tendon with a low level of telopeptide lysyl hydroxylation, aldimine cross-links predominate. The first and most common intermolecular lysine-aldehyde cross-link, dehydrohydroxylysinonorleucine, involves the reaction of the lysyl-aldehyde with a helical hydroxylysine. Dehydrolysinonorleucine (deH-LNL) is formed following reaction with a helical lysine residue. These aldimines are in equilibrium as Schiff bases, which under physiological conditions are effective intermolecular cross-links (725) (Fig. 1.30). The second group of immature cross-links is formed when the telopeptide lysine is hydroxylated. The hydroxylysyl aldehyde derived from this residue reacts with the ε-amino group of a helical hydroxylysine to form the Schiff base, which then spontaneously undergoes the Amadori rearrangement to form the cross-link hydroxylysino-5-ketonorleucine (HLKNL). The keto-imine is stable to acid and thus accounts for the insolubility of bone and cartilage collagens, even at the fetal stage (Fig. 1.31). In bone and other calcifying tissues, a cross-link may also form between an hydroxylysine aldehyde and a helical lysine (725). The tetravalent cross-link, histidino-hydroxymerodesmosine (HHMD), is formed from the reaction of the carbon–carbon double bond of the intramolecular aldol condensation product with histidine and its free aldehyde with the ε-amino group of hydroxylysine. These reactions convert the intramolecular aldol, which has no role in the mechanical properties of the fiber, to a tetravalent intermolecular cross-link, which would have a significant effect on the mechanical properties of the fiber.

Both type I and type III collagens possess the same cross-links in young and old skin. In fetal skin, they possess the keto-imine cross-link when lysyl-hydroxylation is high, but as this reduces postnatally, the aldimine predominates

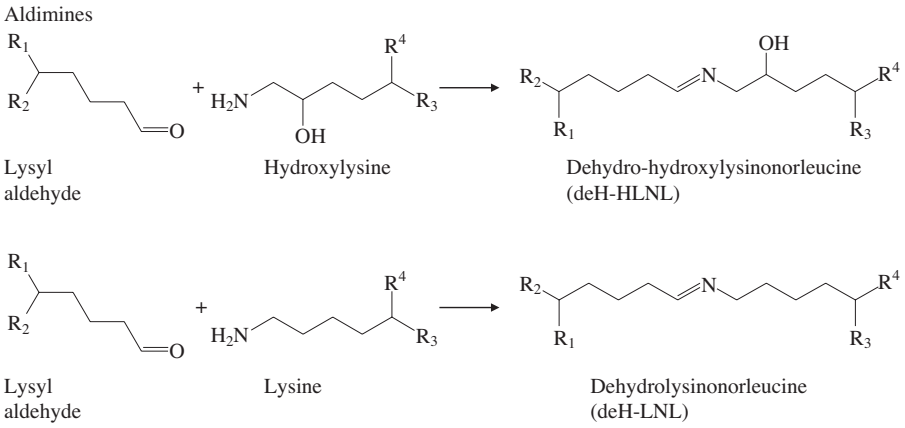



**FIG. 1.30** In this figure, the formation of immature cross-links is depicted. The shown aldimines (deH-HLNL and deH-LNL) are formed by telopeptide lysyl aldehydes that are able to react with triple helical lysine or hydroxylysine (according to Bailey et al., Mech. Ageing Dev. 106: 1–56, 1998).

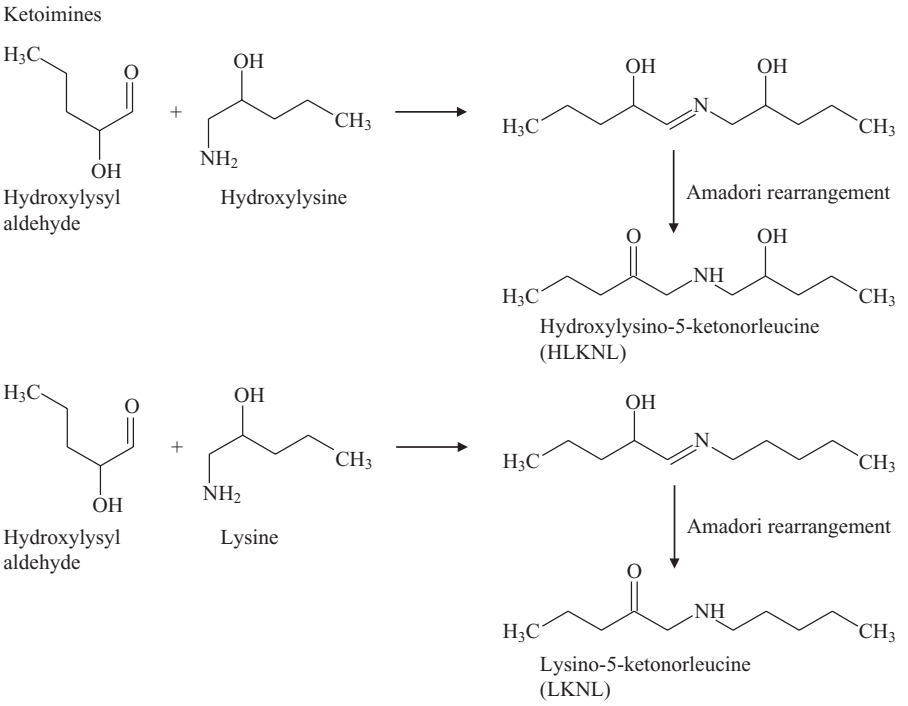



**FIG. 1.31** The ketoimines HLKNL and lysino-5-keto-norleucine (LKNL) are formed from telopeptide hydroxylysyl aldehydes reacting with a triple helical lysine or hydroxylysine (according to Bailey et al., Mech. Ageing Dev. 106: 1–56, 1998).

in both types. This major cross-link, dehydro-hydroxylysinonorleucine (deH-HLNL), is converted to histidino-hydroxylysinonorleucine (HHL) during maturation of human and bovine skin. However, it is interesting to note that old rat skin, rat tail tendon, and mouse skin do not contain HHL despite the fact that deH-HLNL decreases with age (740). A further complication to the maturation of skin collagen is the effect of photoaging. Although HHL, the major cross-link, is stable under UV light, exposure to sunlight has been reported to impede HHL formation, and as such sun-damaged skin is more soluble than unexposed skin (741). Although bone is less hydroxylated than tendon, skin or cartilage, the immature cross-links are primarily derived from the telopeptide hydroxylysyl-aldehydes, the major cross-link being the keto-imine HLKNL.

Collagen provides the basic structural properties of the most vulnerable tissues, such as renal basement membrane, the cardiovascular system, and retinal capillaries, and the most serious late complications of aging involve the modifications of the collagen, which result in the dysfunction of these tissues. The typical age-related changes are accelerated in patients with diabetes due to hyperglycemia and are the major cause of premature morbidity and mortality in these subjects. Glycation can affect the properties of collagen in a number of ways, for example, its optimal biomechanical functioning in the diverse tissue forms, its ability to form precise supramolecular aggregates, the alteration of its charge profile and hence its interaction with cells, and, additionally, glycated collagen, which can act as an oxidizing agent. The most damaging effects are believed to be caused by the formation of glucose-mediated intermolecular cross-links. These cross-links decrease the critical flexibility and permeability of the tissues and reduce turnover (725). Long-term glycation of fibrous collagen occurs during aging and renders it less soluble, more resistant to enzymes and less flexible *in vivo*, and similar reactions occur following incubation with glucose *in vitro* (742). The process is accelerated in diabetic subjects due to the higher glucose levels (743) and in experimentally diabetic animals (744). These effects on the properties of collagen are consistent with formation of the intermolecular cross-links between the collagen molecules within the fiber. This would involve reaction between α-amino groups of lysine in the triple helical parts of the collagen molecule rather than the globular ends of the molecules, as in the case of enzyme-generated cross-links, and consequently lead to more rapid stiffening and enzyme resistance of the collagen fiber. The nonfibrous basement membrane type IV collagen not only becomes stiffer due to increased cross-linking (745), but as a consequence, also results in increased permeability and reduced endothelial attachment (746). The formation of intermolecular cross-links within the collagen fibers and basement membranes are obviously consistent with the decreasing elasticity of retinal capillaries, renal glomeruli, and arterial vessel walls, which are characteristic effects of aging.

In view of the low yields of fluorescent cross-links observed during *in vitro* and *in vivo* investigations, Bailey and his colleagues (747) turned their

attentions to a detailed analysis of nonfluorescent components associated with highly cross-linked collagen and identified a nonfluorescent component, nonfibrillar collagen-1 (NFC-1), the presence of which could be correlated to a change in physiochemical properties consistent with increased cross-linking. The concentration of NFC-1 present in tissue (NFC-1/collagen molecule) and its increase with age suggested that it could be an important intermolecular cross-link. More recent evidence has shown that NFC-1 is a complex consisting of imidazolones derived from GO and methyl glyoxal reacting with arginine and an as yet uncharacterized high-molecular-weight component; the latter is believed to be the cross-link moiety (748).

Glycation has in fact been shown to interfere with cell–matrix and collagen–matrix interactions. For example, glycation of basement membrane alters its binding characteristics with heparin and laminin (749), and fibronectin (750) and glycation of the cell binding domain of type IV decreased endothelial adhesion and spreading (746). Similarly, platelet aggregation and adhesion to collagen are known to be dependent on the quaternary structure of the collagen (751) and involve specific domains in the molecule. The modification of arginine residues in collagen by carbonyl compounds results in the formation of the respective imidazolones (748). GO reacts to form either No-(4-oxo -5-dihydroimidazol-2-yl)-L-ornithine (748) or 1-(4-amino-4-carboxybutyl)-2-imino-5-oxo-imidazolidine (752), whereas MG forms a 4-imidazolon-2-yl derivative existing in three tautomeric forms (748, 753) (Figs. 1.32 and 1.33). An intensive treatment reduced the skin collagen glycation in type 1 diabetes

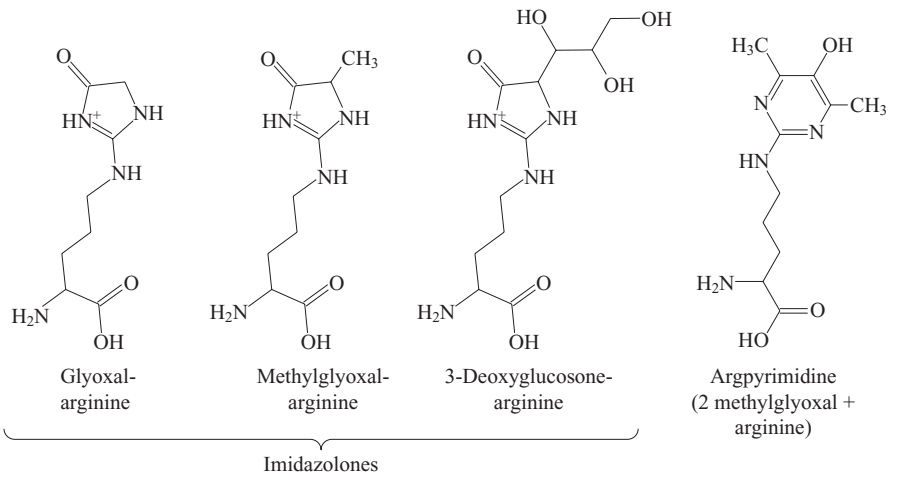
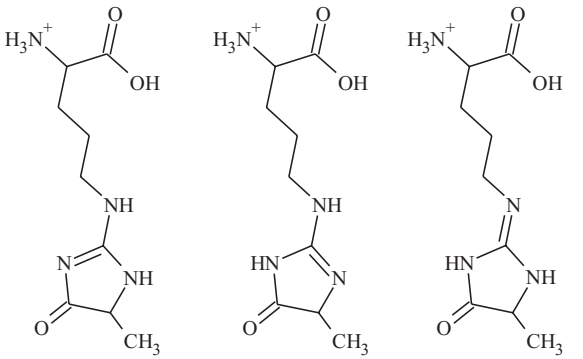
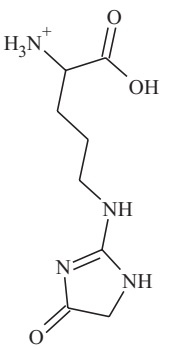


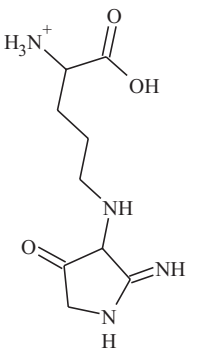

**FIG. 1.32** Structures of different imidazolones resulting from the modification of arginine residues by different carbonyl compounds (first three structures) and the reaction product of arginine with two molecules of MG (argpyrimidine, right structure) (according to Bailey et al., Mech. Ageing Dev. 106: 1–56, 1998).



Three tautomeric forms of an 4-imidazolon-2-yl derivative (βNFC-1)



$N^{\delta}$-(4-oxo-5-dihydroimidazol-2-yl)-L-ornithine (an αNFC-1)



1-(4-amino-4-carboxybutyl)-2-imino-5-oxo-imidazolidine (an αNFC-1)

**FIG. 1.33** NFC-1 structures (NFC, nonfluorescent putative cross-link). The upper row shows the structure of NFC-1, an advanced glycation end product that is derived from the reaction of ribose with protein. In this case βNFC-1, a 4-imidazolon-2-yl that exists in three tautomeric forms. The bottom row shows the structure of two αNFC-1 molecules (according to Paul et al., Biochem. J. 330: 1241–1248, 1998).

(592). Moreover, long-time caloric restriction reduced collagen glycation as shown by Sell et al. (754).

Enzymatic and nonenzymatic intermolecular cross-links are most likely the basic molecular mechanisms of collagen aging and changes in mechanical properties of collagen. The enzymatic mechanism involves divalent aldimine intermolecular cross-links derived from the reaction of aldehydes, which then mature to trivalent cross-links and further stabilize the collagen fiber and is now well known. Recent studies have demonstrated that the rate of turnover and level of telopeptide lysyl hydroxylation modifies the nature of

the cross-link and hence the mechanical strength of the fiber. The slow turnover of mature collagen subsequently allows accumulation of the products of the adventitious nonenzymatic reaction of glucose with the lysines in the triple helix to form glucosyl lysine. These products are subsequently oxidized to a complex series of AGEs, some of which are intermolecular cross-links between the triple helices, rendering the fiber too stiff for optimal functioning of the collagen fibers, and consequently of the particular tissue involved. The glycation reactions following maturation are true aging processes, and attempts at their specific inhibition involve competitive inhibition of the Maillard reaction and chemical cleavage of the glycation cross-links (617).

The enzymatically formed cross-links have been shown to be rather tissue than species specific. The differences should be considered in the major tissue types: skin, bone, and tendon, all predominantly type I collagen. The critical importance of cross-linking is demonstrated by a complete absence of mechanical strength of the collagen fiber following inhibition of the cross-linking enzyme lysyl oxidase. Although PGs and glycoproteins may play a role in the alignment of fibers, there is no evidence that they play a role in their mechanical properties. The nature of the cross-links in bone collagen depends on the activity of two types of enzyme, the lysyl hydroxylases and lysyl oxidases. Three lysyl hydroxylases have been identified to date and there is evidence that one of these is specific for the C-telopeptide of bone collagen (755) and one for the N-telopeptide (756), the other isoform presumably being related to the hydroxylation of the lysines in the triple helix, although additional isoforms may yet be identified. Following extracellular aggregation of the molecules into fibers, the telopeptide lysines and hydroxylysines are oxidatively deaminated by lysyl oxidase. Five isoforms of lysyl oxidase have been identified to date, but their specific function has not been elucidated (757). However, the different amino acid sequences at the amino and carboxy terminals readily suggest one possibility, and the other isoforms may be specific for particular collagenous tissues.

The level of hydroxylation of the telopeptide lysines of type I collagen in skin is very low; consequently, the oxidative deamination by lysyl oxidase leads predominantly to lysine aldehyde. The reaction of this lysyl aldehyde with a specific hydroxylysine within the triple helix leads to the formation of an aldimine or Schiff base, deH-HLNL, which is chemically and thermally unstable, thus accounting for the ready solubility of skin collagen. However, this divalent cross-link does maintain the mechanical stability of the fibers under physiological conditions. With increasing age/maturation, the level of the cross-link decreases (758). This was later shown to be because of reaction with a histidine residue on an adjacent molecule to form the trivalent cross-link HHL (759). As the level of HHL increases, the fibers become less soluble and physically stronger. In certain tissues, two lysyl aldehydes may react to form an aldol condensation product (760), but this is an intramolecular cross-link and would not therefore play a role in determining the physical strength of the fiber. The tetravalent HHMD derived from the aldol is believed to be an

artifact of the borohydride reduction (761). The turnover of fetal skin is high and the telopeptide highly hydroxylated, giving rise to the keto-imine cross-link HLKNL, which disappears in infancy. The turnover of mature skin collagen is much slower than bone collagen, being estimated at about 10–15 years as determined by the extent of β-aspartyl racemization and isomerization. The variation in cross-links with age can give a qualitative indication of turnover; high HLNL levels indicate new collagen synthesis and hence higher turnover, while HHL indicates mature collagen with low turnover. In wound healing, the whole process is repeated from the initial synthesis of HLKNL. Similarly in fibrotic tissue, there are high levels of both HLKNL and HLNL.

In young growing bone, the turnover of collagen is high and the concomitant high levels of lysyl hydroxylases lead to almost complete hydroxylation of the telopeptide lysines. The telopeptide hydroxylysine aldehyde reacts with an ε-amino group of a hydroxylysine in the triple helix to form the initial cross-link HLKNL, binding two molecules head to tail. This is an intermediate divalent cross-link in young bone but provides sufficient strength for the growing bone. The HLKNL then reacts with another hydroxylysine aldehyde to form the trivalent pyridinoline cross-links. The resultant hydroxylysyl pyridinoline (HP) and lysyl pyridinoline (LP) have been used as markers of bone collagen turnover (762). In situations where the lysyl hydroxylase activity is lower, the telopeptide lysines are not completely hydroxylated, leading to the reaction of a lysyl aldehyde with the HLKNL, this time resulting in a pyrrole derivative (763). Hydroxylation of the lysines in the triple helix is also variable in bone, which can lead to LP, the ratio of the two pyridinolines varying with the particular bone. Similarly, hydroxylysyl-pyrrole and lysyl-pyrrole may be formed, but have not been isolated to date (764). The pyrrole appears to be concentrated at the N-terminal, whereas pyridinolines are present at both termini but with the LP predominantly at the N-terminal (765). Recent studies have shown that there is a correlation between the bending stress of compact bone with the pyrrole rather than the pyridinoline cross-link (766, 767). In the case of cancellous bone, the strength and stiffness by compression testing correlated with the HP/LP ratio, the higher the ratio the stronger and stiffer the bone (768). Further studies revealed that a high ratio of pyrrole to pyridinoline correlates with the architecture of the cancellous bone (769). With increasing age, there is a significant loss of bone in both males and females, resulting in a decrease in mechanical strength, but the composition of the bone collagen, as determined by the rate of turnover, level of hydroxylation, and nature of the cross-links, remains constant (770).

The telopeptides of type I tendon collagen contain both lysine and hydroxlysine-aldehyde residues, leading to the formation of both HLNL and HLKNL, the relative proportions of each varying with the particular tendon examined. Consequently, with increasing age, both pyridinoline and HHL become the major cross-links in mature tendons and account for the age-related increase in mechanical strength. Skin and tail tendons from mice and rats are different in that telopeptide hydroxylation is very low and

consequently they possess only deH-HLNL, which, although decreasing as the animal matures, does not form HHL (771).

The collagen of articular cartilage is highly hydroxylated, both within the triple helix and the telopeptide, resulting in the formation of HLKNL and HP as the major cross-links. The type II collagen is decorated on the surface with type IX collagen, and it was suggested that the Col 1 domain of type IX occupies the whole region of the type II fiber, and that the Col 2 domain links to a molecule in an adjacent type II fiber, thereby forming a stabilizing network throughout the articular cartilage (772).

The second process involved in the age-related changes in the physical properties of collagen and elastin is the reaction with tissue glucose. The discovery of the reaction of glucose with the α-amino end group of the terminal valine in hemoglobin (773) and with the ε-amino group of peptide-bound lysine, which increased with age (774), led to new areas of research on the effects of these glucose reactions in aging. The higher levels of glucose in diabetes mellitus similarly led to rapid glycation and cross-linking of collagen, thereby increasing the mechanical strength and stiffness above the normal, thus reducing its optimal efficiency (775). The reaction is nonenzymatic, primarily with the side chains of lysine and arginine. The initial reaction is the formation of a Schiff base followed by a spontaneous Amadori rearrangement. The reaction with glucose occurs through the open chain form, hence it is slow as the equilibrium changes from the cyclic pyranose form. Other sugars react faster, for example, ribose and MG, but are present in minute quantities in tissue. Both the Schiff base and the Amadori product subsequently undergo oxidation, particularly in the presence of metal ions and ROS, and may fragment to more reactive species such as deoxyglucosone, as well as GO and methylglyoxal (MGO), both of which react rapidly with lysine and arginine. The reaction with the side chains of lysine and arginine alters the charge profile of the fibers which in the future may prove to be just as important as cross-linking. For example, the modification of the arginine side chain could affect binding to the Arg-Gly-Asp (RGD) site involved in the recognition of two matrix integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$, which form the physical link between cell and matrix (776).

MDA, as a dialdehyde, is obviously capable of cross-linking collagen, and the close proximity of unsaturated lipids and collagen in the vascular system (777) strongly suggested the importance of the observed stiffening of the large arteries in diabetes (778). *In vitro* incubation of collagen with MDA rapidly induces insolubility and mechanical brittleness in the fiber, indicating extensive cross-linking. It was shown that the MDA and acetaldehyde react to give a potential cross-linking compound, dihydropyridine (779), not a double Schiff base as was the previously favored reaction.

Odetti et al. (780) evaluated the modification of proteins due to glycation and to side products of lipoperoxidation by measuring their specific fluorescence levels in the collagen of 65 healthy Wistar rats during the aging process. The relationships among the fluorescence at different wavelengths were also

reported. An increase of all fluorescence intensities was observed in rat collagen during aging: the glycation-related ones $\lambda_{ex} = 370/\lambda_{em} = 440$ nm and $\lambda_{ex} = 335/\lambda_{em} = 385$ nm and HNE adduct-related $\lambda_{ex} = 356/\lambda_{em} = 460$ nm were exponential, whereas that derived from MDA adducts was almost linear $\lambda_{ex} = 390/\lambda_{em} = 460$ nm. The *in vitro* study confirmed a good specificity of collagen fluorescence after incubation with a reducing sugar (0.5 M ribose for 6 h) for protein glycation, and after incubation with MDA (0.1 mM for 3 h) for lipoperoxidation adducts; surprisingly enough, HNE (0.5 mM for 3 h) significantly increased the fluorescence related to pentosidine-like products ($\lambda_{ex} = 335/\lambda_{em} = 385$ nm), suggesting that this compound might be the precursor of products with a fluorescence similar to pentosidine or of pentosidine itself.

### 1.4.2 Elastin

Elastin is a connective tissue protein found in virtually every tissue and organ of mature animals and is a major constituent of elastic tissues in an ECM such as blood vessels, lungs, and ligaments. Elastin is the core protein of the elastic tissue contributing to the elastic property of the vascular wall, the skin, and the lung (781). Several diseases that occur in elastic tissues are due to alterations in the function of elastin. Elastin is primarily synthesized during the development of various cells such as vascular smooth muscles and fibroblasts via a soluble precursor, tropoelastin. After secretion into the extracellular space, the tropoelastin is rendered insoluble by cross-link formation by lysyl oxidase in the posttranslational modification step. Lysyl oxidase catalyzes the oxidative deamination of the ε-amino group of lysine residues in elastin and collagen to form α-amino-adipic γ-semialdehyde (allysine). Once generated, these allysine residues spontaneously condense with other ε-amino group lysine residues to form inter- and intramolecular covalent cross-links such as desmosine and isodesmosine (Fig. 1.34) (782). The principal steps of elastin biosynthesis have been characterized, and alterations in the pathway have been implicated in a number of disease states, such as atherosclerosis, pulmonary emphysema, pseudoxanthoma elasticum, and diabetes mellitus (782–783). Elastin also undergoes progressive changes during senescence and has been associated with age-related diseases (630, 784). Fluorescent substances are present in elastin and the fluorescence of elastic fibers increases with age. A variety of cross-linking amino acids have been isolated from elastin hydrolysates and identified (785, 786), although none of them have been identified to be the major fluorophore of elastin yet. A new cross-linking amino acid named cyclopentenosine (CP) (787) was detected, which has fluorescence similar in many respects to the compound of the major fluorophore in elastin. The cross-link was proposed to have a 2-cyclopenten-1-one skeleton and to be derived from three allysine residues. Cyclopentenone and 2-cyclopenten-1-one are known to have important biological functions such as growth inhibitory activity. CP was abundantly detected in elastin-rich tissues such as nuchal

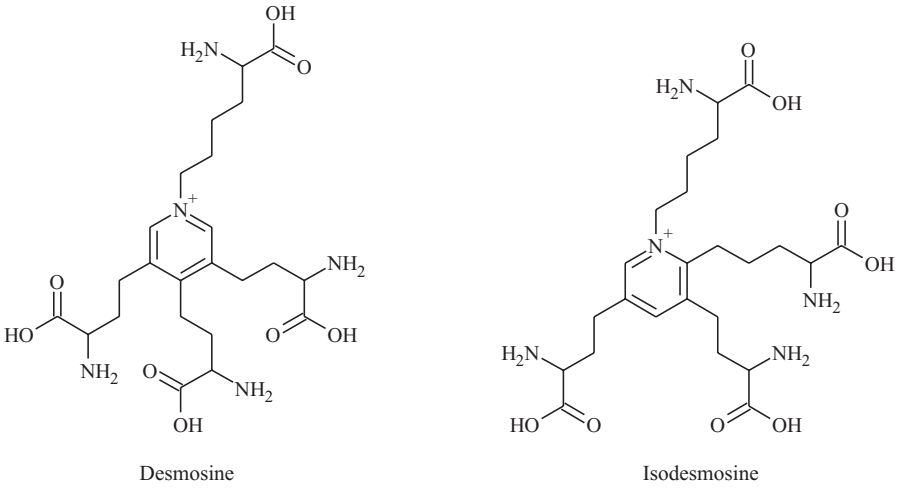



**FIG. 1.34** Desmosine (left structure) and isodesmosine (structure on the right), two inter- or intramolecular cross-links, formed by reaction of allysine and lysine residues (according to Ma et al., Proc. Natl. Acad. Sci. U.S.A. 100: 12941–12943, 2003).

ligament, aorta, lung, and spleen, and was also found in collagen-rich tissues such as Achilles tendon and bone, suggesting that CP exists in both elastin and collagen. In dermis, which is highly composed of elastin and collagen tissue, the CP level was the lowest among the other tissues. This reduction might be due in part to the decomposition of the CP cross-link by UV radiation. Akagawa et al. (782) examined the age-related changes in the concentration of CP in the aorta from rat (short-lived species) and bovine (long-lived species). The CP content was very low in the newborn rat but increased markedly with growth. After maturity, the CP content remained nearly the same or slightly decreased. In bovine aorta, the CP content scarcely changed from 7 months to 16 years.

Elastin peptides (EPs) are mostly generated during vascular aging and the atherosclerotic process. They induce free-radical and proteases production from cells, which are the major components of the atherosclerotic process. Fulop et al. (781) investigated whether the interaction between EPs and neutrophils as well as monocytes contributes to LDL oxidation. The EP as well as the active epitope, the hexapeptide VGVAPG, is able, in a differential concentration and time dependence, to induce the oxidation of LDL. EP is able to induce, via the production of free radicals by neutrophils, the oxidation of LDL very rapidly and in higher concentration compared to monocytes. These effects of EP are occurring through the stimulation of the 67 kDa elastin-laminin receptor (ELR).

The interaction between EPs and cells was shown to be mediated by a cell membrane ELR, composed by three subunits. Among them is a 67-kDa subunit

mediating the binding of soluble EPs with the elastin receptor, identified by Hinek et al. (788) as a lectin reacting with lactose.

In skin after chronic exposure to sunlight, especially in persons with a fair complexion, hyperplasia of the elastic tissue is usually evident in the upper dermis by the age of 30. These changes found in the photoaged skin are called actinic elastosis (641). Mizutari et al. (641) used a monoclonal anti-AGE antibody (6D12), whose epitope is CML, and demonstrated that the lesions of actinic elastosis were modified by CML. Further, immunohistochemical and immunoelectron microscopic examination with 6D12 demonstrated that CML accumulates predominantly in elastic fibers, especially in the photo-induced degenerated area. They conclude that UV-induced oxidation may accelerate CML formation in actinic elastosis of photoaged skin.

### 1.4.3 The Oxidation of Membrane Proteins

Membrane proteins are critical for the maintenance of biological systems and may be important targets in the oxidation process. The results obtained from electrophoresis of proteins of erythrocyte membranes treated with chloroderivatives indicate the formation of intra- and/or intermolecular protein disulfide bonds. Thus, there are reasons to suggest that disulfides are important products of thiol group oxidation by N-chloroamino acids and HOCl/$OCl^-$ in the erythrocyte membranes. Effect of action of chlorocompounds on erythrocyte membrane proteins was the loss of acetylcholinesterase activity. Inhibition of the enzyme was due to reduction of maximal velocity ($V_{max}$). The decrease of acetylcholinesterase activity and $V_{max}$ was not accompanied by alterations in the enzyme affinity for the substrate. Another consequence of treatment of erythrocyte membranes with chloroderivatives is the depletion of free protein amine groups observed for the highest concentrations of AlaCl, LysCl, and SerCl employed (789).

### 1.4.4 Band 3

The SCA was discovered in 1975 (790). It appears on old erythrocytes and acts as a specific signal for the termination of erythrocyte life by initiating the binding of IgG autoantibody and subsequent removal by phagocytes. The aging antigen itself is generated from the band 3 protein. The binding of anti-band 3 autoantibodies is the result of band 3 aggregation in the plasma membrane of senescent erythrocytes (344, 791).

A protein immunologically related to band 3 was demonstrated in many other cells (792). Interestingly, band 3 is also present in the nuclear (792), Golgi (793), and mitochondrial membranes (794) Band 3 was also found in the kidney and the nervous system (795, 796). The autosomal recessive neurological disease, choreoacanthocytosis, is associated with band 3 abnormalities (797).

The presence of an 80-kDa serine protease in the oxidized erythrocyte membranes that preferentially degrades oxidized proteins, namely oxidized protein hydrolase (OPH) (798–800), was demonstrated. OPH showed both endopeptidase activity for oxidized proteins and exopeptidase activity for acylated short chain peptides. It was found that this enzyme is identical with acylpeptide hydrolase (ACPH) (800). OPH is originally present in the cytosol and becomes adherent to the membranes under oxidative conditions. During oxidation. several glycoproteins, including band 3, aggregate on the cell surface (801). This formation of aggregates of the carbohydrate chains of band 3 protein is the important step in the marking of erythrocytes for removal, due to their recognition by anti-band 3 autoantibody (334, 802) and macrophages (803).

The band 3 protein function include anion exchange and acid–base balance, ankyrin binding, structural stability, and integrity (804). Age-related band 3 malfunction was reported for all these functions (805, 806).

SCA-band 3 were also detected in old but not young brains (807). This presence is well structured and found to be cell type specific (796). Hemichromes possess a strong affinity for band 3 cytoplasmic domain and, following their binding, lead to band 3 oxidation and clusterization. Those band 3 clusters show increased affinity for naturally occurring antibodies (NAbs), which activate, complement, and finally trigger the phagocytosis of altered RBCs (808).

Band 3, comprising 25% of the total RBC membrane protein, appears to be the major target of NAbs (338, 344, 804). Band 3, also termed the anion exchanger (AE1), appears to be structured in two independent domains: the membrane-spanning domain catalyzes the anion exchange (mostly $HCO_3^-$/$Cl^-$) and contains the antigenic determinants recognized by NAbs; the cytoplasmic domain binds to different proteins: cytoskeletal proteins (ankyrin, protein 4.1, protein 4.2), glycolytic enzymes (aldolase, GAPDH, phosphofructokinase), hemoglobin denaturation products (hemichromes), and to the protein tyrosine kinase ($p72^{syk}$). Each of these interactions appear to have a central effect for the structure and the functions of RBCs (809). NAbs appears to bind only to modified band 3, in particular, strong evidence indicates that band 3 clusters induced by hemichromes are always associated to intense NAbs binding (810, 811).

Band 3 in tissues such as brain performs the same functions as in erythrocytes, including the formation of the SCA. Band 3 ages as cells and tissues age. Studies, to date, indicate that the anion transport ability of band 3 decreases in brains and lymphocytes from old mice. This decreased transport ability precedes obvious structural changes such as band 3 degradation and generation of SCA, and is the earliest change thus far detected in band 3 function. Other changes include a decreased efficiency of anion transport (decreased $V_{max}$) in spite of an increase in the number of anion binding sites (increased $K_m$), decreased glucose transport, increased phosphorylation, increased degradation to smaller fragments as detected by quantitative binding of antibodies

to band 3 breakdown products and residue 812–830, and binding of physiologic IgG autoantibodies *in situ* (807).

Bosman measured the aging-related parameters in erythrocytes from individuals with sickle cell anemia. Most sickle erythrocytes were shown to have characteristics that are also found in senescent normal erythrocytes, such as an increased density and considerable concentrations of cell-bound IgG. Together with the concomitant changes in structure and function of band 3, the data suggested that most sickle erythrocytes have undergone a process of accelerated aging (812). Bosman and Kay found that neither incubation with the free radical-generating xanthine oxidase/xanthine system, nor treatment with MDA, an end product of free radical-initiated LPO, results in age-specific changes of band 3 (797).

Recently, to overcome the difficulties to obtain homogeneous fractions that contain RBC of the same age, because of the smaller mean corpuscular volume of senescent RBC compared with young cells, a percoll gradient has been used for the separation. A higher carbonyl content as well as an increase in fragmentation of band 3 protein was observed in senescent RBC subpopulations compared with young cells (813).

### 1.4.5 Actin

Actin is perhaps one of the most functionally diverse proteins in eukaryotic cells. Traditionally the actin cytoskeleton is thought of as playing structural and organizational roles as well as being a direct participant in a wide range of motility processes. Recently actin has been found to be involved in many nuclear processes as well, including regulation of chromatin structure and transcription (814–816). This functional diversity makes the actin cytoskeleton an ideal candidate for integrating signaling between diverse cellular processes. The actin cytoskeleton is an early target of cellular oxidative stress (817), and in certain disease conditions, the oxidative state of actin in the cell is very different from normal. For example, in sickle cell crisis, a major factor that contributes to the inflexibility of irreversibly sickled cells (ISCs) is the formation of an intracellular disulfide bond between C284 and C373 of β-actin (818, 819). It is found that oxidoreductase called Oye2p (old yellow enzyme) regulates oxidation between C285 and C374 in *Saccharomyces cerevisiae* suggests that actin oxidation takes place in all eukaryotic cells and that the actin cytoskeleton is subject to redox regulation (820).

The effects of ROS on tryptophan residues in α-skeletal actin and troponin I (fast skeletal muscle isoform) using an established rat model of acute oxidative stress induced by X-ray irradiation was tested. In the control samples (no oxidative stress), the single Trp residue of troponin I (position 161) and the four tryptophan residues present in actin (positions 79, 86, 340, and 356) were only oxidized at very low levels. Postirradiation, the level of oxidized versions increased for most positions within 3 h. Tryptophan residues located inside the

proteins, however, required longer time periods. In the mass spectra, the following oxidation products of tryptophan were detected: kynurenine; oxolactone; hydroxytryptophan or oxindolylalanine (isobaric); hydroxykynurenine; dioxindolylalanine, N-formylkynurenine, or dihydroxytryptophan; and hydroxyl-N-formylkynurenine. Despite a partial recovery after 24 h, the degree of oxidation of all oxidized versions was still higher than in the control samples (821). In the same model, the content of reactive carbonyl groups increased fourfold within the studied 24 h period. Among the five Cys residues of actin, Cys(239) and Cys(259) were oxidized to sulfenic (Cys-SOH), sulfinic (Cys-SO(2)H), or sulfonic (Cys-SO(3)H) acids by increasing amounts over the time periods studied. The content of methionine sulfoxides also increased for 15 of the 16 methionine residues, with Met(44), Met(47), and Met(355) having the highest sulfoxide contents. Met(82) was also further oxidized to the sulfone (822).

## 1.5 MECHANISM AND FACTORS INFLUENCING THE FORMATION OF PROTEIN OXIDATION PRODUCTS

Basic mechanisms involved in the oxidation of proteins by ROS were clarified using ionizing radiation of amino acids, peptides, and proteins (823). One of the primary reactions leads to hydrogen atom abstraction (Fig. 1.35, reaction

| | |
|---|---|
| $\sim NHCRHCO\sim + {}^{\bullet}OH \rightarrow \sim NHC^{\bullet}RCO\sim + H_2O$ | **Reaction 51** |
| $\sim NHC^{\bullet}RCO\sim + O_2 \rightarrow \sim NHCOO^{\bullet}RCO\sim$ | **Reaction 52** |
| $\sim NHCOO^{\bullet}RCO\sim + HO_2^{\bullet} \rightarrow \sim NHCOOHRCO\sim + O_2$ | **Reaction 53** |
| $\sim NHCOOHRCO\sim + HO_2^{\bullet} \rightarrow \sim NHCO^{\bullet}RCO\sim + H_2O + O_2$ | **Reaction 54** |
| $\sim NHCO^{\bullet}RCO\sim + HO_2^{\bullet} \rightarrow \sim NHCOHRCO\sim + O_2$ | **Reaction 55** |
| $H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$ | **Reaction 56** |

**FIG. 1.35** Hydrogen atom abstraction from the protein polypeptide backbone after reaction with an hydroxyl radical ($^{\bullet}OH$), resulting in the formation of a carbon-centered radical (**reaction 51**) that forms peroxyl radicals by addition of an $O_2$ molecule (**reaction 52**). The formed peroxyl radical is turned into an alkyl peroxide by reacting with the protonated form of superoxide (hydroperoxyl, $HO_2^{\bullet}$) (**reaction 53**). Further reactions of the alkylperoxide with protonated superoxide ($HO_2^{\bullet}$) yield an alkoxyl radical (**reaction 54**) and a hydroxyl derivative (**reaction 55**). The Fenton reaction provides hydroxyl radicals ($^{\bullet}OH$) in a cellular environment (**reaction 56**) (according to Stadtman, Curr. Med. Chem. 11: 1105–1112, 2004).

51); the formed radical attracts molecular oxygen (Fig. 1.35, reaction 52). The formed peroxyl radical afterward reacts with superoxide, protonated in physiological conditions (Fig. 1.35, reaction 53). The alkylperoxide might interact further with the superoxide radical (Fig. 1.35, reaction 54) and finally form a hydroxylated carbon (Fig. 1.35, reaction 55). Alternatively the carbon-centered radical formed in reaction 1 might form -C-C- cross-linked proteins; in addition, other pathways for the peroxyl radical and the alkoxyl radical exist. The Fenton reaction (Fig. 1.35, reaction 56) might induce the formation of the hydroxyl radical, starting with reaction 51, but $Fe^{2+} + H^+$ can replace $HO_2^\bullet$ in reactions 53–55 (823). Interestingly, alkoxyl radical derivatives of proteins are able to undergo peptide bond (3).

However, exposure of proteins to ionizing radiation leads to β-scission of amino acid side chains. For example, β-scission of alanine, valine, leucine, and aspartic acid residues leads to the generation of free formaldehyde, acetone, isobutyraldehyde, and glyoxylic acid, respectively. In each case, cleavage of the side chain leads to the formation of a carbon-centered radical (~NH$^\bullet$CHCO~) in the polypeptide chain, as occurs when glycine residues undergo $^\bullet$OH-dependent α-hydrogen abstraction (29).

The rate of protein degradation is governed in part by the equilibrium concentration of unfolded protein; in addition to that, increases and decreases in degradation rates may be due to factors that modulate the unfolding equilibrium. Chemical modification may shift the distribution toward a higher concentration of unfolded species, thereby explaining the facile degradation *in vivo*. At least nine of such chemical modifications that facilitate proteolytic attack are known. These chemical modifications are phosphorylation of serine and threonine residues; formation of mixed disulfide derivatives of Cys residues; carbamylation of lysine residues; oxidation of nonheme iron sulfur centers; conjugation of ε-amino groups of lysine with ubiquitin; oxidation of methionine to methionine sulfoxide and methionine sulfone; deamidation of glutamine (Fig. 1.36) and asparagine residues; and glycosylation and oxidation of amino acids by mixed-function oxidase systems (824).

As another aspect, oxidation of proteins can lead to hydroxylation of aromatic groups and aliphatic amino acid side chains, nitration of aromatic amino acid residues, nitrosylation of sulfhydryl groups, sulfoxidation of methionine residues, chlorination of aromatic groups and primary amino groups, and conversion of some amino acid residues to carbonyl derivatives. Oxidation can also lead to cleavage of the polypeptide chain and to formation of cross-linked protein aggregates. Furthermore, functional groups of proteins can react with oxidation products of PUFAs and with carbohydrate derivatives (glycation/glycoxidation) to produce inactive derivatives (825).

### 1.5.1 Redox Status

At high concentrations, free radicals and radical-derived, nonradical reactive species are hazardous for living organisms and damage all major cellular

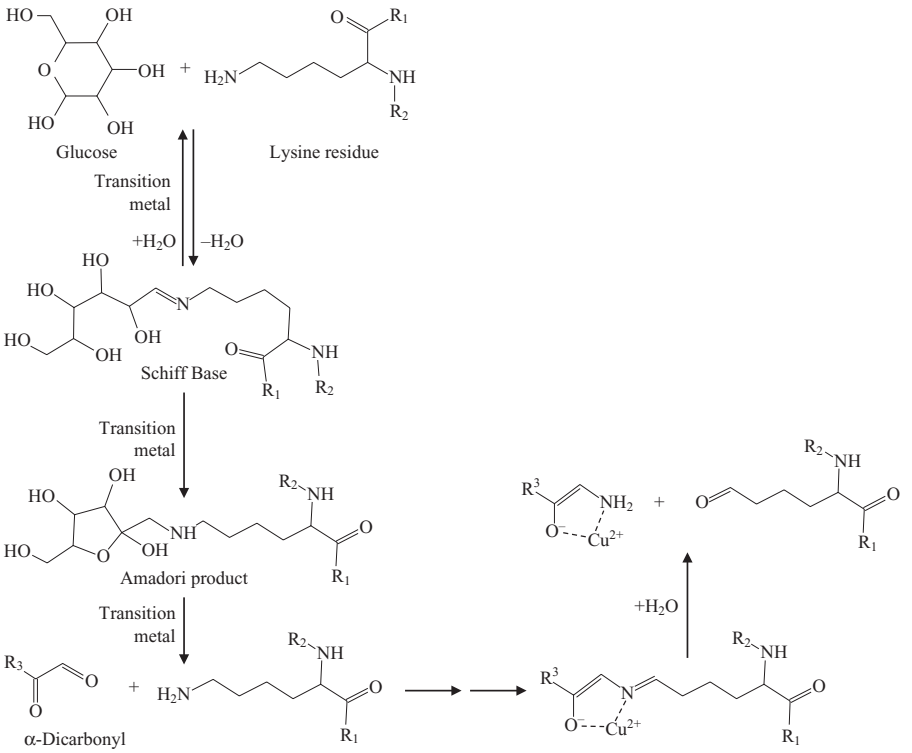



**FIG. 1.36** Oxidative deamination of a lysine residue by Strecker-type reaction (according to Akagawa et al., Eur. J. Biochem. 269: 5451–5458, 2002).

constituents. At moderate concentrations, however, nitric oxide (•NO), superoxide anion, and related ROS play an important role as regulatory mediators in signaling processes. Many of the ROS-mediated responses actually protect cells against oxidative stress and reestablish "redox homeostasis" (128).

A major mechanism of redox homeostasis is based on the ROS-mediated induction of redox-sensitive signal cascades that lead to increased expression of antioxidative enzymes or an increase in the Cys transport system, which in turn facilitates in certain cell types the increase in intracellular GSH. Cells or tissues are in a stable state if the rates of ROS production and scavenging capacity are essentially constant and in balance. Redox signaling requires that this balance is disturbed, either by an increase in ROS concentrations or a decrease in the activity of one or more antioxidant systems (128).

In 2000, Smith et al. (826) have shown that extracellular effectors such as thyroid hormone, bone morphogenic protein 4, basic fibroblast growth factor, and platelet-derived growth factor (PDGF) regulate the cellular redox state of rat glial precursor cells. An intracellular redox environment induced to favor a more oxidized state by thyroid hormone or bone morphogenic protein

4 leads to a differentiation of the precursor cells into oligodendrocytes or astrocytes.

The balance between the production of ROS and the maintenance of antioxidant defense systems is considered essential to the protection of the cellular redox status. If the amount of free radicals/reactive particles in a cell overwhelms its antioxidative capacity, that cell is in a state of oxidative stress (827, 828). Kim et al. (829) investigated the age-related redox status of serum by analyzing hydrogen peroxide, hydroxyl radical, superoxide-scavenging abilities, and other redox markers. The results clearly indicated that the serum redox balance shifted toward oxidation during aging.

The phrase redox regulation conjures a variety of definitions: Alterations in the ratio of NADH to $NAD^+$ and NADPH to $NADP^+$ regulate function by providing substrate and product control of enzyme-catalyzed oxidation and reduction reactions. Formation or reduction of disulfide bonds is critical to protein folding and function. More recently, redox regulation has come to signify control of transcriptional and enzymatic events by free radicals (128, 830–832). The concept of redox regulation provides an additional facet to the free-radical theory of aging.

Proteins containing reactive sulfhydryl groups have the potential to undergo reversible redox regulation (830, 832) and irreversible oxidative modification and inactivation (833–835). Redox regulated proteins play critical roles in metabolism, cell signaling, and transcriptional control (128, 830, 832), and irreversible oxidation of enzymes is likely to have a critical influence on metabolism (836). Therefore, changes in the redox status might influence the cellular metabolism via protein modifications, leading to age-related changes and disease via various signaling events (837). Blanco et al. concluded that Cys/CySS and GSH/GSSG redox states in human plasma undergo variation with an increased magnitude of variation in Cys/CySS redox state in older persons. This variation could alter sensitivity to oxidative stress over a course of hours (838).

Redox mechanisms function in regulation of cell growth, and variation in redox state of plasma thiol/disulfide couples occurs in various physiological conditions, including diabetes, chemotherapy, and aging. Jonas et al. (839) tried to determine whether a systematic variation in extracellular thiol/disulfide redox state ($E_h$) over a range (from 0 to –150 mV) that occurs in human plasma altered proliferation of cultured cells. Experiments were performed with a human colon carcinoma cell line (Caco2), which grows slowly in the absence of serum and responds to peptide growth factors with increased rate of cell division. The extracellular redox states were established by varying concentrations of Cys and cystine, maintaining constant pool size in terms of Cys equivalents. Incorporation of 5-bromo-2-deoxyuridine (BrdU) was used to measure DNA synthesis and was lowest at the most oxidized extracellular $E_h$ (0 mV). Incorporation increased as a function of redox state, attaining a 100% higher value at the most reduced condition (–150 mV). Addition of insulin-like growth factor 1 (IGF-1) or epidermal growth factor (EGF) increased the rate of BrdU incorporation at more oxidizing redox conditions

(from 0 to –80 mV) but had no effect at –150 mV. Cellular GSH was not significantly affected by variation in extracellular $E_h$. In the absence of growth factors, extracellular $E_h$ values were largely maintained for 24 h. However, IGF-1 or EGF stimulated a change in extracellular redox to values similar to that for Cys/cystine redox in plasma of young, healthy individuals. The results show that extracellular thiol/disulfide redox state modulates cell proliferation rate and that this control interacts with growth factor signaling apparently independently of cellular GSH (839).

Many proteins present on cell surfaces and located in extracellular fluids contain Cys and methionine residues that are subject to oxidation. These proteins, which include transporters, receptors, and enzymes, respond to variations in the extracellular thiol/disulfide redox environment. Changes in activity of these proteins can alter the ability of organs to function normally and influence processes such as nutritional absorption, secretory function, neurotransmission, and susceptibility to toxicants. In addition, extracellular redox state can regulate tissue homeostasis through effects on cell proliferation, differentiation, apoptosis, and immune function. Consequently, extracellular redox state can have important influences on health status and disease states and thus could be a target for nutritional interventions (543).

A complex interaction exists between extracellular and cellular redox control, with extracellular redox ultimately dependent upon the cellular redox state. Two principal systems maintain cellular thiol/disulfide redox state: GSH and Trx. GSH is a low-molecular-weight thiol present at millimolar concentrations in cells, whereas Trx is a small protein present at micromolar concentrations. GSH is well suited for functions in detoxifications, interorgan Cys homeostasis, and redox control. Trx contains a dithiol motif at its active site, which is ideally suited for reduction of protein disulfides, sulfoxides, and sulfenic acids, but is also capable of peroxide elimination and is used for deoxyribonucleotide biosynthesis. Cellular GSH/GSSG redox state varies in cells in association with proliferation, differentiation, and apoptosis. Proliferating cells have GSH/GSSG values ranging from –260 to –230 mV (840, 841). The mechanistic link between redox and cell cycle has not been completely elucidated, but a redox effect has been identified. For instance, in normal fibroblasts, mRNA levels of *gro*, a gene associated with proliferation, were correlated with the redox environment and the proliferative state of the cells (842). The GSH pool becomes oxidized (from –220 to –190 mV) during growth arrest, either due to differentiation or contact inhibition. In normal fibroblasts, an increase in confluency led to an oxidation of the redox state (+34 mV) and cessation of proliferation, whereas fibrosarcoma cells maintained a reduced state and continued to proliferate despite an increased culture density (843). In human bronchial epithelial cells, growth arrest followed a decrease in intracellular GSH and Cys levels (844). An oxidation of intracellular GSH/GSSG redox as well as lowered intracellular GSH and GSSG concentrations were observed in contact-inhibited, nondividing human retinal pigment epithelial (hRPE) cells (845). These nondividing hRPE cells were more susceptible to

tert-butyl hydroperoxide (tBH)-induced apoptosis. During apoptosis, GSH export from cells is activated and the redox state is further oxidized to between –170 and –150 mV (846, 847). A Cys deficiency limits cell growth and is sufficient to result in a marked oxidation of the GSH/GSSG redox pair (848). This oxidation indicates that dietary availability of sulfur amino acids may directly determine thiol/disulfide balance in cells. Because Cys is required for protein synthesis, the associated redox change may provide a central mechanism for coupling dietary sulfur amino acid availability to cell growth and tissue homeostasis. As mentioned, cell surface proteins such as receptors, transport proteins, and enzymes that contain thiol moieties can be influenced by redox regulation. Cell-surface glycosylphosphatidylinositol-anchoring proteins and lipid raft moieties have been suggested to be direct targets of oxidative stress. Oxidation of proteins in these rafts induces clustering through disulfide bond cross-linking of cell surface proteins and consequent activation of Src family protein tyrosine kinases (849). Iesaki and Wolin (850) found that extracellular thiol oxidation activates a redox-regulated coronary vasodilator mechanism that involves the inhibition of $Ca^{2+}$ influx. Treatment of isolated endothelium-removed bovine coronary arteries with the thiol oxidant diamide caused relaxation, presumably due to the obstruction of $Ca^{2+}$ influx. Protein tyrosine phosphatases are intracellular redox-sensitive proteins that can be reversibly oxidized and act as regulators of cell surface receptor tyrosine kinases (851). N-Acetylcysteine (NAC), Cys, and GSH have the ability to modulate the redox state of extracellular Cys residues of target proteins, but may also exert effects intracellularly by acting upon the redox-sensitive phosphatases. In Caco-2 cells, MAPK p44 phosphorylation was dependent on modulation of the extracellular cysteine/cystine (Cys/CySS) redox state, where in the most reduced conditions, MAPK p44 was phosphorylated. NAC, Cys, and GSH addition to bovine and human chondrocyte serum promoted survival through phosphorylation of extracellular signal-regulated kinases (ERKs) (852). Several studies are available to show that extracellular thiol/disulfide redox state becomes more oxidized with aging. Among the changes that are well documented are decreased GSH, increased CySS, and increased total homocystine, which is predominantly present in disulfide forms. GSH concentration in the cerebral spinal fluid of humans also decreases with age (542, 853). In older subjects, compared with younger subjects, whole-blood GSSG was increased and in the plasma GSH was lower and the GSH/GSSG redox was more oxidized (854). There was also a decrease in Cys and an increase in CySS concentration. Oxidation of the Cys redox pool correlated with a decrease in body cell mass and plasma albumin, which suggests that such an oxidation may contribute to the loss of cell mass (548, 855). However, therapeutically, albumin levels in humans increased with NAC treatment, indicating loss of albumin can be rectified (855, 856). Most of the diseases for which extracellular thiols or extracellular thiol disulfide redox state could be important are chronic and complex diseases, usually dependent upon multiple factors. Currently available information indicates that at least five dietary

components contribute to plasma thiol concentrations and/or redox state. These include the intake of sulfur amino acids, the availability of glutamine, the presence of dietary antioxidants, the content of inducers of GSH synthesis, and the adequacy of other redox active micronutrients such as selenium, flavin, and niacin.

GSH, in its reduced form, and GSSG can be analyzed in a variety of ways, including highly specific and easy-to-do assays (857). Since the total redox capacity is more important than the total amount of reduced GSH, often the percentage of oxidized GSH, which is given as GSSG/(GSH + 2GSSG), is measured. From the concentrations of GSH and GSSG, the half-cell reduction potential ($E_{hc}$) can be calculated according to the method of Schafer and Buettner (858).

PBN, a so-called spin-trapping compound, reacts with free radicals and yielding in a stable nitroxyl product. PBN offers protection from and traps free radicals during ischemia–reperfusion-mediated injury to heart and brain of experimental animals (859). It reduced the formation of protein oxidation products and the inactivation of enzymes during aging in various models (15, 32).

Age-related redox changes in blood plasma have been observed by a decrease in the total thiol levels and levels of serum GSH (860). Aging was shown to have also an influence on NAD+ metabolism in the heart, lung, liver, and kidney of female Wistar rats, which was parallel with an increase in protein carbonyl and o-,m-tyrosine formation (861).

### 1.5.2 Protein Turnover

The cellular regulation of the protein turnover is complex. Conventionally, protein modifications brought about by ROS and RNS are considered as random and highly undesirable, leading to protein malfunction. In turn, these modified proteins must be removed to preserve cellular function, as they are inappropriately damaged by random free-radical action. In addition to that, $^{\bullet}NO$/peroxynitrite and superoxide anion/$H_2O_2$ play an important role in the normal regulation of protein turnover (124).

Besides the changes in protein degradation, a decline in protein synthesis during aging was described. In order to find out the possible mechanism responsible for the *in vivo* protein synthesis decline during aging, Parrado et al. determined the effect of aging on the various steps of protein synthesis. Their results showed that the elongation is the most sensitive step to aging. The tests on the EF-2, the main protein involved in the elongation step, showed that this protein has a higher content of carbonyl groups and is less active in old rats. In addition, the molecular mass analysis of EF-2 showed that this protein becomes fragmented in old rats (40).

Cellular metabolism is in a state of dynamic equilibrium; individual proteins vary greatly in their half-lives, ranging from minutes to months, and as such they are being continually synthesized and degraded by highly regulated

processes. The rate of turnover of some proteins has been correlated with their amino acid N-terminal residue sequences (N-end rule pathway), which apparently favor ubiquitination of the protein and its subsequent proteasomal digestion. It has been reported that N-terminal Cys of certain proteins must first be arginylated in order to be degraded in a ubiquitin-dependent pathway. However, arginylation of N-terminal Cys requires its prior oxidation, a process that is controlled and rate limited by $^{\bullet}NO$ and oxygen (862).

In general, the level of a modified protein is always the result of the balance between the relative rates of protein modification and degradation. For example, oxidized proteins may accumulate in muscle tissues due to the slower repair or degradation (863). Some mechanisms of protein aging, that is, racemization of aspartyl residues (647) or nonenzymatic glycosylation (601), occur slowly and are therefore found predominantly in long-lived proteins.

Correlations between protein structure and degradation rates have been thought to explain the basis of protein turnover. Degradation rates have been correlated with thermal stability, dissociation of stabilizing ligands, and susceptibility to proteolytic cleavage (824). Bachmair et al. (864) suggest that the rate-limiting step in the degradation of long-lived proteins is slow aminopeptidase cleavage, which exposes a destabilizing amino acid. The destabilizing element is rapidly recognized and leads to degradation according to the N-end rule. Other observations are not consistent with a simple proteolytic mechanism of degradation. For example, acidic proteins are generally degraded more rapidly than are neutral or basic ones, the rate of degradation is nearly proportional to the amount of apolar surface area of the folded protein, and proteins composed of large polypeptide chains are degraded more rapidly than those composed of small chains. It was proposed that proteins that are rapidly degraded in eukaryotic cells contain regions rich in proline (P), glutamic acid (E), serine (S), and threonine (T)—the PEST sequence (865). The PEST hypothesis appears to be consistent with observations that acidic proteins are generally degraded more rapidly than basic proteins, as PEST proteins tend to be acidic.

### 1.5.3 Metal-Catalyzed Oxidation (MCO)

Since 1981, Stadtman and colleagues have examined the inactivation of proteins in cell-free systems involving the metal ion-catalyzed autoxidation of ascorbate and/or hydrogen peroxide; in some cases, the metal ions were derived from metalloproteins. These systems are now termed "metal-catalyzed oxidation systems" instead of the previous confusing term "mixed-function oxidation systems" (44).

Under normal conditions, the MCO systems are the major source of oxidative damage, requiring hydrogen peroxide or organic hydroperoxides (8) and a transition metal (61) modifying preferentially amino acid residues at the metal-binding site (254, 866).

MCO systems catalyze the conversion of lysine residues to α-aminoadipic semialdehyde and arginine and proline residues are oxidized to glutamic semialdehyde, and these oxidations account for 40–100% of the carbonyl derivatives formed by MCO oxidation of purified proteins (29). The sensitivity of protein His residues toward MCO *in vitro* has been known for years (867). Surprisingly, little analytical data are available that stable His oxidation products, specifically 2-oxo-histidine, are formed and can accumulate *in vivo*. Moreover, the site-specific nature of the metal-catalyzed reactions has been confirmed by the results of studies with *E. coli* GS, which show that inactivation of the enzyme involves the conversion of $His^{269}$ to an asparagine and of $Arg^{344}$ to a glutamic semialdehyde derivative. Moreover, these His and Arg residues are both situated at one of the two metal-binding sites at the catalytic center of the enzyme. Susceptibility of the enzyme to degradation by the multicatalytic protease is also associated with the modification of a second His residue ($His^{209}$ or $His^{210}$), which is situated at the second metal-binding site on the enzyme (866). An inactivation of GS was shown to be influenced by its adenylation state, which also regulates the enzyme and some multienzyme cascades (868).

In metal ion-catalyzed oxidations, the location of the active transition metal ion becomes important, and residues such as His, Cys, Lys, and Met, which bind metal ions, may localize reactions to their vicinity. The differential capacities of proteins to bind metal ions (render them either redox inactive (e.g., transferrin, lactoferrin) or active) also influences the distribution of damage among protein populations (44). Selective damage to particular residues present in peptides and proteins can arise from the binding of a metal ion or other initiating species at a particular site on a peptide or protein (869). Evidence has been presented for the formation of radicals at specific sites on a number of proteins, including β-amyloid precursor protein (870), iron regulatory protein-2 (871), and mitochondrial $F_1$-ATPase (872), as well as small peptides (50, 873). In each case, discrete, well-defined fragments were detected, and this has been ascribed to complexation/binding of the metal ion to particular sites, and subsequent generation of $^{\bullet}OH$ or other highly reactive species which would be expected to react in their immediate vicinity, thereby inducing site-selective damage. Fibrinogen appears to be the plasma protein that is most sensitive to *in vitro* metal ion-catalyzed carbonyl formation (874).

Iron chelators (EDTA, o-phenanthroline) inhibit the inactivation of all enzymes tested except creatine kinase (253) and G6PDH. Chelators actually stimulate the oxidation of the latter two enzymes, presumably because they possess a binding site for the iron–chelate complex. Carcinogenic nickel compounds are practical examples of MCO, which are actively phagocytized by target cells of transformation, allowing Ni ions to reach high levels in the cell and producing genetic damage associated with carcinogenesis (875). Nickel ions have been shown to form oxygen radicals and to increase oxidants in cells catalyzing the formation of covalent cross-links of proteins and amino acids to DNA (876, 877). Ni binds very tightly with proteins, compared to DNA; this

fact may explain the selectivity of nickel interaction with protein-rich heterochromatin (878). Costa (875) demonstrated that Ni produced increased protein oxidation as determined by carbonyl formation. Ni also increased oxidation of cellular proteins *in vitro* in the presence of hydrogen peroxide. This increase in oxidants formed in cells could be responsible for the variety of intracellular effects caused by nickel. One effect attributed to increased oxidants is the covalent cross-linking of certain amino acids, such as histidine, Cys, and tyrosine. These cross-links are covalent since they resist extraction with sodium dodecyl sulfate (SDS). Their covalent character and resistance to EDTA extraction negates the direct participation of $Ni^{+2}$, suggesting that Ni caused the cross-linking of these amino acids by a catalytic mechanism rather than participating directly in the cross-links.

### 1.5.4 Heat Shock Proteins

Chaperones are important in controlling correct folding after protein synthesis (879). Chaperones among the HSP families that are induced during oxidative stress and may protect against the irreversible denaturation of a partially unfolded molecule. Thus macrophage colony-stimulating factor, which enhances $O_2^{\bullet -}$ output, induces the synthesis of HSPs of 60, 70, and 90 kDa, and confers enhanced resistance to $H_2O_2$; the chaperonins may contribute to this effect (880) and protect against autoxidative damage during the respiratory burst. A parallel induction of chaperones arises on exposure of the intracellular facultative bacterium *Francisella tularensis LVS* to $H_2O_2$; this is presumably a defense against oxidative stress produced by the host macrophages (881). Many human cells also respond to oxidative stress by the induction of HSPs (e.g., see (882)). Complexes of partially unfolded proteins with chaperones may trigger further responses in these systems.

HSPs represent a very conserved family of cytoprotective proteins that are specifically induced in response to several environmental stress at the cellular level (heat shock, cellular energy depletion, oxidative stress, or inflammation) (883). Among these proteins, the inducible form of the 70-kDa family, that is, HSP70, plays an important role in cytoprotection by preventing abnormal folding of newly synthesized polypeptides, or by assisting in the repair of damaged proteins or in the degradation of irreversibly damaged proteins (884, 885). An adapted stress response in terms of transcription and translation of HSP70 is necessary to ensure cell survival under stressing conditions (886). Clear evidence has demonstrated in aging animals an altered basal expression of HSP70 in various types of tissues and an altered ability to synthesize these proteins in response to environmental stress (883, 887). Elderly people seem to be characterized by a diminished basal expression of HSP70 and a blunted induction in response to different stress like hyperthermia. This decline in cellular expression and induction of HSP70 in aging mammalians could be implicated in the pathogenesis or in the worsening of various diseases observed during the aging process (888).

On the molecular level, it was shown that the acquired tolerance is accounted for by accumulation of the major stress response protein, HSP70, and other HSPs. Under normal physiological conditions, stress is usually elevated gradually, and cells develop acquired tolerance while stress is still mild; it protects cells at later severe stages of stress. Thus, stress-inducible HSP70 expression, which is responsible for acquired stress tolerance, represents one of the major cellular protective systems. However, this line of defense is being progressively weakened and lost with aging. Aging is characterized by a progressive impairment in the ability to adapt to environmental changes. Manifestation of this impairment at the cellular level is the age-related attenuation of HSP70 expression and the consequent loss of the ability to develop acquired tolerance. Attenuation of HSP70 expression was shown in cells isolated from young organisms and aged in culture, in cells isolated from aged human donors, and in aging animal model systems. It should be noted that the intrinsic sensitivity of cells to stresses does not change during aging; what changes—because of attenuation of HSP70 expression—is the ability to form the acquired tolerance and consequently the ability to resist stresses. The physiology of aged cells changes in such a way that HSP70 loses its cytoprotective role and consequently its expression is attenuated so as not to waste cellular resources. That aged cells cannot express HSP70 due to unspecified damages typical for aged cells, for example, chromosomal damage in the HSP70 gene region, was disproved by a demonstration of treatment of aged cells with a proteasomal inhibitor MG132 (889) or with the HSP70-inducing factor (HIF) (890). HIF induces HSP70 expression at levels comparable to those seen in young stressed cells. These results demonstrated that age-related attenuation of HSP70 expression can be reversed, and stress resistance can be restored in aged human cells. Oxidative and other stress conditions can induce the synthesis of further copies of these stress proteins, principally via the action of heat shock transcription factors (HSF). There appears to be a relationship between the presence of altered proteins and transcription of chaperone proteins due to the fact that HSF is normally bound to the major chaperone proteins HSP90 and HSP70, or to itself in an inactive form. But when the HSP90/HSF complex encounters an aberrant protein, the latter binds to the complex and displaces the HSF, thereby enabling it to bind to heat shock transcription elements in the DNA and stimulate synthesis of more chaperone proteins. The resulting increase in chaperone protein numbers presumably enables the cell to maintain delivery of altered proteins to the proteasomes for destruction and thereby prevent formation of deleterious protein aggregates (59).

The ER provides another site for control of protein quality. Here, folding occurs cotranslationally in three phases: the nascent chain enters ER lumen, folding takes place after release of the completed chain from the ribosome, and finally protein oligomerization occurs; high concentrations of chaperone and folding proteins are located in the ER lumen to facilitate correct folding and final assembly (891). It appears that many of the ER-associated chaperones are similar to those found in the cytoplasm (e.g., HSP40, HSP70, and

HSP90), although there are some different ones, too (calnexin, calreticulin, and thiol–disulfide oxidoreductases). Nevertheless, the outcome of unsuccessful folding is the selective degradation of the aberrant polypeptide via ERAD (ER-associated degradation), which involves the retrotranslocation of the unfolded polypeptide to the cytosol for ubiquitination and destruction by the proteasome. It appears that in some cases the proportion of the newly synthesized protein that fails to satisfy the selection criteria is surprisingly large; for example, 40% of the γ-opioid receptor fails to mature and is degraded (891). Various heat shock or stress proteins protect organisms and cells against aging in general (892–894) and the toxicity of altered proteins in particular (895) to some degree.

The various forms of altered proteins should not be considered independently, as one form of protein aberration appears to predispose toward other types of postsynthetic change. For example, erroneous synthesis increases a polypeptide's potential toward oxidative damage, which in turn can raise its tendency for deamidation (12). Additionally, denaturation can increase a protein's glycation potential (896). Cross-linking of proteins can inhibit proteolytic elimination by the proteasomes (897, 898) and increase in the production of age-related protein aggregates (899, 901); such an accumulation of protein aggregates might lead to chaperone binding.

## 1.6 PROTEIN AGGREGATES: FORMATION AND SPECIFIC METABOLIC EFFECTS

Aggregation of oxidized proteins might be driven by a multitude of covalent or noncovalent (as hydrophobic and electrostatic) interactions (902). Covalent interactions might be reversible, as the often occuring -S-S- formation, or irreversible, as the formation of 2,2′-biphenyl cross-links (15).

Protein aggregates are formed as oligomeric complexes of misfolded or unfolded proteins which do not normally interact with each other. They are mainly insoluble and metabolically stable under normal physiological conditions (903). The aggregate is independent from the original structure of the protein and introduces a new toxic element into cellular metabolism. It was considered by Fabunmi et al. (904) that approximately 30% of the newly synthesized proteins are misfolded. This number can increase by the presence of mutated proteins, or under conditions that favor secondary unfolding; such as oxidative stress (905, 906). These misfolded proteins, like those in neurodegenerative diseases, have a strong tendency to aggregate (907). It might require several steps depending on the nature of the initial conditions, leading to unfolding and aggregate formation. Generally, due to the complex process of specific intermolecular interactions, such as in the physiological aging, the process of aggregation is slow (15, 908, 909). The process depends on the concentration of the protein, the intracellular conditions, and supporting factors, such as those described for tau aggregate formation (910). In so-called ordered

aggregation (911), polymerization is not significant until a certain critical concentration of the monomer is reached.

Kopito suggests the process of aggregation to be a controlled process (912). In certain cases, this requires an active, retrograde transport of misfolded proteins on microtubuli (903, 913), although this transport seems to be limited to some specialized forms of protein aggregate formation. Furthermore, one has to take into account that aggregate formation is a continuous process, possibly accompanied by a time-dependent enlarging of the aggregate. Secondary modification reactions might take place, such as cross-linking, oxidation, AGE formation, or ubiquitinylation. Other cellular proteins might bind to the protein aggregate due to its chemically and biologically highly reactive surface. This, for example, was described for the proteasome, which tends to bind to tau aggregates (914). In senescent cells, a colocalization of the proteasome with lipofuscin was found. One of the best studied aggregating proteins is the amyloid peptide, which seems capable of inducing free-radical production in AD (915). The amyloid peptide, as other aggregates, is able to bind metals, and these can produce radicals through the Fenton reaction (916–918). Since aggregation of proteins is also promoted through metals, a spiral of aggregation steps starts (919). A fairly broad spectrum of protein aggregate formation initially occurs not due to covalent cross-links, but because of new hydrophobic and electrostatic interactions (920–924). This aggregated material can afterward be further modified by a great variety of cellular metabolites, including aldehydic LPO products (923, 925). Bifunctional aldehydes, like 4-HNE or MDA, are able to form covalent cross-links (925). This material might undergo further reactions and form the age pigment lipofuscin, also called AGE pigment-like fluorophores by various authors (926). The formed aggregates are comprising a major part of the cellular hydrophobic phase, as demonstrated by costaining with the lipophilic dye ANEPPS (927). The involvement of free radicals, and cross-linking reactions by aldehydic LPO products or carbohydrates, has been postulated by several groups as one of the initial steps in the formation of fluorescent oxidized/cross-linked aggregates (923, 928).

The occurrence of protein aggregates in cells may trigger a number of intracellular reactions, including the fact that the aggregates might act to promote cell death (917, 927). Most protein aggregates are ubiquitinylated. Interestingly, the accumulation of intracellular ubiquitin conjugates leads to cell cycle arrest (929). Furthermore, while the proteasomal system is inhibited by aggregates, regulatory proteins and transcription factors cannot be degraded in time, and thus may initiate the apoptosis pathway (930). Therefore, a disturbance in the normal level of certain proteins can cause the induction of apoptosis.

The question of the relationship between proteasome inhibition and protein aggregates was raised in a number of studies (931–933). It was reported that heavily oxidized and cross-linked proteins are poor substrates for the proteasome. More than that, these aggregates are able to inhibit the proteasome as

shown (718, 925, 934). Furthermore, proteasome activity drastically declines in cells fed with aggregated/oxidized proteins (934). Proteins covalently aggregated with cross-linkers such as HNE cross-linked proteins are also able to inhibit the proteasome (718, 925). It was demonstrated that HNE cross-linked Aβ peptide, which forms the senile plaques of AD, is able to inhibit the proteasome (935). Whereas in our hands neither the amyloid peptide nor HNE alone were able to affect proteasome activity, the high-molecular-weight amyloid peptide HNE aggregates were effective inhibitors of the proteasome. Mutated proteins tending to aggregation are also able to inhibit the proteasome, such as the mutant ataxin-1 (936) or the Huntingtin protein with an expanded polyglutamine repeat (937). The age-related increase of age pigments was demonstrated in the 1970s by Strehler et al. in human myocardium (938) and by Reichel et al. in rodent brain (939). Later on this pigment was called "lipofuscin," "ceroid," or "AGE pigment-like fluorophores" by various authors, indicating the involvement of carbohydrates in final fluorophore formation. It is believed that all these pigments have the same principal origin (926), although there might be tissue-specific differences (926, 940–942). The involvement of cross-linked protein oxidation products (688) and of free radicals in the formation of fluorescent oxidized/cross-linked aggregates has frequently been postulated (943). The accumulation of protein aggregates and inhibition of the proteasome seem to be much more dramatic in postmitotic cells (944), possibly explaining the vulnerability of neurons. Obviously in dividing cells, a dilution of the aggregated proteins is constantly under way, preventing the accumulation of high levels of protein aggregates.

Several diseases are accompanied by the accumulation of cross-linked proteins. This accumulation of oxidized protein aggregates takes place either extracellularly or within several cellular compartments. Due to the formation and location of these aggregates in various cell types and several parts of the body, differences in the effects of these protein aggregates on the function of cells are expected. In several cases, aggregated/cross-linked material will undergo autophagocytosis, resulting in a major accumulation of the material in lysosomes (945, 946).

### 1.6.1 Accumulation of Oxidized Proteins

The accumulation of oxidized proteins is one of the key factors in the aging process. Oxidized proteins are normally repaired or degraded by the proteasomal system. This system is the most important intracellular protein degradation machinery, responsible for the degradation of oxidized proteins. For unknown reasons, the removal of oxidized proteins is disturbed in aged cells. This leads to the accumulation of nonfunctional proteins (947). The accumulation of altered proteins, which accompanies aging, may be a consequence of an increase in their production via oxidation and nonenzymatic glycosylation and/or a decrease in the cellular ability to selectively degrade them (59). In general, protein degradation protects a cell against the toxic accumulation of

abnormal proteins and proteins that are no longer necessary. This is of pharmacological evidence because of the increasing number of potential protein drugs such as EGF, hepatitis B vaccine, human growth hormone, human insulin, interferons, and malaria vaccine (824).

Intracellular protein breakdown protects the cell against the toxic accumulation of abnormal polypeptides arising because of missense mutations, mistakes in RNA or protein synthesis, incorporation of amino acid analogues, posttranslational modifications, or intracellular denaturation. Accumulation of altered proteins might be partially the consequence of a reduced protein degradation (948). It was found that half-lives of proteins introduced into hepatocytes (949, 950) and pulse-labeled proteins (951) were increased with age. Moreover, the half-life of ovalbumin in cells isolated from old animals was higher compared with young (950).

Another consequence of the accumulation of protein aggregates is the induction of the stress response, possibly in an attempt of the cell to rescue itself from the toxic structures. Should the response be insufficient to effect a total clearance of the aberrant structure, then the cell might become permanently stressed. This could be fatal because a second consequence of the stress response is the shutdown of the synthesis of regular housekeeping proteins. It is conceivable that under these conditions cell death could result from a failure to replace housekeeping proteins necessary for continued viability (59).

There is increasing evidence showing that proteolytic activity declines with age in many cell types. Both cytosolic proteasomal and lysosomal activities have been reported to decline. Much evidence is accumulating which indicates that proteasomal dysfunction may be a cause of the age-related accumulation of altered proteins (898, 952). Explanations for the lowered activities are mostly related to the inhibitory effects of incompletely degraded and cross-linked peptide species exert on proteasomes. Lipid protein cross-links in the form of lipofuscin (the so-called age pigment) may inhibit lysosomal activity (900, 901). Therefore, an age-dependent decline in the lysosomal system functionality was proposed to contribute to aging (899).

D-Amino acids are catabolized by D-amino acid oxidase and D-aspartate oxidase. These enzymes therefore have a detoxifying role regarding the D-amino acids, which may have accumulated during aging (953).

Mitochondria, as the primary intracellular energy source, use oxygen as the final substrate in the ATP production process. Interestingly, in aged cells, an increasing number of enlarged and giant mitochondria were observed. This enlargement may be caused by accumulated damage in mitochondria, impairing, on the one hand, the mitochondrial fission, and on the other hand, the degradation of these enlarged mitochondria. It is assumed that organelles, including mitochondria, are degraded within the lysosomes. The uptake into the lysosomal compartment takes place by a process called macroautophagy (954, 955).

A variety of diseases and physiological processes are characterized by the intra- or extracellular accumulation of proteins. These often cross-linked

protein aggregates do not have a common terminology. Among others, the terms "protein aggregates," "plaques," "inclusion bodies," or "aggresomes" are used (956). Johnston et al. (903) defines an aggresome as "a pericentriolar, membrane-free, cytoplasmic inclusion containing misfolded, ubiquitinated proteins ensheeted in a cage of intermediate filaments formed specifically at the microtubule organization center (MTOC)." The term "inclusion body" was used in a somewhat broader definition that does not include the microtubule dependence (903). The term "protein aggregate" appears to have a rather wide specificity, mainly requiring the existence of aggregations of misfolded protein. For extracellular protein aggregates, the term "plaque" is more common. The terms "ceroid" and "lipofuscin" are, in general, used to describe protein material that accumulates during the physiological and/or pathological aging process. In a broader sense, this describes accumulated intracellular protein materials that are also oxidized and modified by secondary reactions.

### 1.6.2 Lipofuscin and Ceroid

It is believed that the cross-linked proteins react further with other cellular components, forming a material referred to as lipofuscin, ceroid, or AGE pigment-like fluorophore by various authors. Lipofuscin is accepted to consist of oxidized protein (30–58%) and lipid (19–51%) clusters (957). Proteins within lipofuscin are linked by intramolecular and intermolecular cross-links. Many of these cross-links are caused by nonproteineous compounds, including oxidation products of other cellular components such as 4-hydoxy-2-nonenal (HNE) (958–962). The formed final product is resistant to degradation by cellular proteolytic systems (963, 964). Interestingly, this insoluble material is not exocytosed by cells (965–967). Many groups have observed that lipofuscin mainly accumulates during the aging of postmitotic cells. Catalytic iron seems to be an important factor in the further oxidation reactions of the initial protein aggregate (917). There has been some speculation about dysfunction of the lysosomal proteases due to the accumulation of lipofuscin in lysosomes, but it is still unclear whether the initial cross-linking reactions are taking place in the cytosol and these aggregates are taken up by macrophagy, or whether lysosomes are required components of the lipofuscin formation (15, 968).

Oxidatively modified proteins are normally repaired or degraded by proteasome and lysosomes and thereby replaced by *de novo* synthesized proteins. The repair of oxidatively modified proteins is restricted to the reversion of few modifications on Cys and methionine residues. The major process to remove oxidized proteins is the degradation by the proteasome. If the oxidative damage is faster than the proteolysis rate, the oxidized proteins accumulate within the cells. In addition to this, the high-molecular-weight aggregates are inhibitors of the proteasome and therefore they enhance their own formation as the removal of damaged proteins slows down (917, 934). Because of their ability to bind transition metals like iron and copper (917), and as lipofuscin

is a fluorochrome, it seems to sensitize lysosomes and cells to blue light. That process might be important for the pathogenesis of age-related macular degeneration (969, 970). A steady increase of accumulation of lipofuscin for postmitotic cells is a known inevitable hallmark of aging, and also some neurodegenerative diseases are linked to elevated levels of proteinous material aggregation.

Because there is no lipofuscin-specific antibody, and the composition of lipofuscin varies between different cell types, using lipofuscin autofluorescence has become one of the most important methods of detection and quantification (970). Other methods include some classical histochemical lipid-staining techniques and agents, such as Sudan black, Nile and Berlin blue, ferric ferricyanide, Fontana–Masson, Ziehl–Neelsen, hematoxylin, eosin, or osmic acid (971). Using the electron microscope, lipofuscin granules are detected as osmophilic, preferentially perinuclear bodies, surrounded by a typical lysosomal membrane. Light and electron microscopical immunocytochemistry reveal lysosomal enzymes associated with lipofuscin. The wide autofluorescence spectrum that is detected by fluorescence or laser scanning microscopy is probably one of the most important properties of lipofuscin, making its quantification possible. Although the nature of lipofuscin fluorophores has not been fully clarified, *in vitro* experiments suggest that reactions between carbonyls (mainly aldehydes resulting from LPO reactions) and amino compounds produce Schiff bases, 1,4-dihydropyridines or 2-hydroxy-1,2-dihydropyrrol-3-ones, that display autofluorescent properties similar to natural lipofuscin (969). As the measurement of the autofluorescence is fast and easy, lipofuscin formation is a good biomarker for age-related impairments in protein turnover by the proteasomal and lysosomal system.

Lipofuscin accumulation is a much more rapid process in postmitotic cells of short-lived species than in comparable cells of long-lived ones (972). This is especially striking when species such as birds and rodents of similar sizes and metabolic rates are compared. Birds generally live much longer and mitochondria produce much less ROS than rodents (973, 974). The higher the amount of intralysosomal redox-active iron, the more Fenton-type reactions are possible. Most probably, this is one of the key factors in lipofuscinogenesis, since lipofuscin formation in model systems of cultured cells can be significantly increased by the addition to the culture medium of small amounts of a hydrated iron phosphate complex, which become endocytosed and enriched the lysosomal apparatus with low-molecular-weight, redox-active iron. Conversely, lipofuscin formation is substantially hampered by allowing cells to endocytose the potent iron chelator desferrioxamine (969).

Lipofuscin is a photosensitizer, especially when excited by blue light (975). Consequently, lipofuscin-loaded retinal pigment epithelial cells (supporters of the photoreceptors) may be irreversibly damaged by light, explaining the increased risk of macular degeneration in aged individuals (969). The retina is in special focus of interest concerning the intracellular lipofuscin distribution. A fluorophore of lipofuscin (A2-E) accumulated in retinal cells not only in

age-dependent macular degeneration but in many pathological processes. This is the focus of interest because of the phototoxicity shown by lipofuscin, respectively synthetic A2-E, releasing free radicals after absorption of blue light. Investigations using LDL coupled to synthesized A2-E showed a localization almost exclusively in the lysosomes, except for a small amount bound to the cell membrane of pigment epithelial cells (976).

Thus, there are strong indications that progressive lipofuscin deposition ultimately decreases cellular adaptability and promotes the development of age-related pathologies, including neurodegenerative diseases, heart failure, and macular degeneration. A number of pathologies are associated with early, age-independent deposition of lipofuscin pigment, which in these cases is occasionally called "ceroid." This may occur as a result of amplified reparative autophagy following, for example, viral or radiation attacks. Intralysosomal degradation may then reach extensive levels and since this process is not perfect, it explains why lipofuscin pigments accumulate. Suppression of the processes through which lysosomes degrade autophagocytosed substances (pharmacologically or due to genetic deficiency of certain lysosomal enzymes in so-called lipofuscinoses) is known to induce the accumulation of lipofuscin pigment as well (977). This happens because in such cases autophagocytosed material degrades only slowly, or not at all, allowing additional time for its oxidation and cross-linking (965). It is also important to realize that lysosomal degradation is counteracted by iron-catalyzed peroxidation and polymerization of macromolecules, explaining why enhanced formation of nondegradable material may be the result of enhanced autophagy, increased oxidative stress, augmented amounts of lysosomal redox-active iron, or reduced capacity of intralysosomal degradation. Although all these conditions accelerate lysosomal pigment formation, its morphology and chemical composition remains similar to that observed in normal aging. Thus, the distinction between "lipofuscin" and "ceroid" appears very obscure, and at least the use of the term "ceroid" only complicates the understanding of lysosomal pathology (970). The nuclear cell compartment is, in contrast to the cytosolic compartment, largely free of lipofuscin (978).

Lysosomes are intracellular compartments with high $H^+$ concentrations which are obtained by ATP-consuming proton pumps and degrade macromolecules enzymatically at acidic pH (approximately 4.5–5.5) (979). Many different degrading enzymes, such as phosphatases, proteases, polysaccharidases and oligosaccharidases, sphingolipid-hydrolyzing enzymes, and lipid-hydrolyzing enzymes, are found in lysosomes, enabling the degradation of almost any cellular structure. In hepatocytes, approximately 300 lysosomes fill approximately 1% of the whole cellular volume (980, 981). The turnover of mitochondria appears to be regulated by lysosomal degradation (982). Due to the high iron content of mitochondria, the uptake of mitochondrial material into the lysosome does not appear to stop the production of free radicals immediately. This postulate was described as the "mitochondrial-lysosomal axis theory of postmitotic cellular aging" (983). According to this theory, the resulting ROS are

able to cause LPO in the lysosomal membrane, accompanied by the formation of cross-linking LPO products, such as HNE and MDA. Most importantly, the oxidation of the lysosomal membrane might lead to a rupture (984), followed by the release of redox-active iron and toxic products into the cytosol, including lipofuscin. Such lipofuscin is either taken up again by the endosomal–lysosomal compartment or disturbs the cytosolic metabolism.

The proteasome appears to be able to recognize unfolded proteins with surface-exposed hydrophobic amino acid patches (985). However, if cytosolic proteins are damaged severely, it is possible that—because of free-radical reaction or the involvement of cross-linking agents, such as HNE—some cross-linked proteins are formed. Intensive studies showed that cross-linked proteins are poor substrates for the proteasome (925, 935, 986). It appears that such proteins cannot be degraded by the proteasome and, furthermore, are able to inhibit the proteasome. This is of special interest because proteasomal inhibition might reduce the turnover of oxidized proteins and facilitate the accumulation of such proteins, which, due to their reactive surface, are able to cross-link. The inhibition of the proteasome seems to depend on the protein aggregate surface structure (987).

Keeping the cytosol clean from consequences of oxidative damage and keeping the protein pool working are functions of the lysosomal and proteasomal systems (988, 989). The capacity of these systems decreases over time in postmitotic aging cells (990, 991), during oxidative stress, in the progress of pathological events (927, 947, 992, 993), or in lipofuscinoses (994, 995), which are lysosomal storage diseases, including Batten's disease (996). Therefore, some oxidatively damaged proteins are not immediately degraded but further oxidized, resulting in the intracellular accumulation of lipofuscin. The iron content of lipofuscin is explained by the absorption of metal-containing proteins, including mitochondrial proteins. In fact, about 50% of the protein content in some lipofuscin might be the remnants of the subunit c of the mitochondrial ATP synthase (997). This results in a redox-active surface of lipofuscin particles, enhancing the Fenton reaction. Therefore, lipofuscin is able to facilitate its own formation because of its ability to drive LPO and protein oxidation (998). Permanently dividing, short-living cells such as bone marrow or mucosa epithelia cells have the ability to dilute the accumulated lipofuscin by cell division (899, 999, 1000). In contrast, postmitotic cells such as neurons, cardiac myocytes, or skeletal muscle cells are not able to do so (969, 1001). Investigations of intracellular accumulation and distribution of lipofuscin showed an increase in postmitotic aged cells compared with young or dividing cells; the nucleus, however, is free of lipofuscin. Comparing tissue samples from several organs (heart, liver, skeletal muscle, lung, kidney, cerebellum, and testes) of rats of different ages (2, 11, and 29.5 months) showed a low amount of lipofuscin in the youngest animals and significant increases, up to 28-fold, in the oldest ones; in some tissues, for example brain, kidney, and lung, the differences between the animals at 11 and 29.5 months were not statistically significant (1002, 1003).

The autofluorescent material is distributed in the perinuclear space in the form of globular structures, having diameters ranging from 0.1 to approximately 5.0 μm. Various patterns of lipofuscin distribution and appearance were observed in every cell and tissue but lipofuscin seemed to be located near the Golgi apparatus (heart) (1004), the perinuclear region (skeletal muscle), or the pericanalicular region, and was surrounded by mitochondria (liver). In copper-exposed hepatocytes, the induced lipofuscin granules were distributed almost homogenously in the cytosol (1005). Another study showed a perinuclear distribution in pyramidal cells (sector CA 1) and a large amount of lipofuscin in the apical site of the pericarion (CA 2, 3, and 4) (1006). Experiments to detect the intracellular organelles containing lipofuscin were performed using intracerebroventricular injection of colchicine into aged mice brains (1007). The result was a transport of lysosomes and lipofuscin from the cell bodies to the dendrites in neurons of various brain regions, resulting in a fivefold increase of lysosomes and sixfold rise of lipofuscin granules in the dendrites (1008). Experiments to determine the lipofuscin distribution in neurons of Alzheimer-diseased brains showed an increased, but largely homogenous, distribution of lipofuscin in the neuronal cytosolic space (1009).

Recently, the release of lipofuscin by RPE cells has been observed in monkeys treated with a small molecule belonging to the tetrahydropyridoethers class. In this direction, therapeutic strategies started to be used to remove lipofuscin from RPE cells, which may have implications for the treatment of age-related macular degeneration (1010).

## 1.7 METHODS TO MEASURE PROTEIN OXIDATION PRODUCTS IN RESEARCH LABORATORIES

Several biomarkers have been identified to provide a measure of oxidative damage to biomolecules, including amino acid oxidation products (methionine sulfoxide, o-tyr and dityrosine, chlorotyrosine and nitrotyrosine), chemical modifications of proteins following carbohydrate or lipid oxidation, such as $N^{\varepsilon}$-CML and $N^{\varepsilon}$-CEL, MDA and 4-HNE adducts to amino acids. These compounds have been measured in different targets such as short-lived intracellular proteins, plasma proteins, long-lived extracellular proteins, and in urine, which allows to monitor tissue-specific damage to proteins in biological systems (622). Although different methods have been developed for determination of protein damage, the techniques *in vivo* are poorly established because the oxidative intermediates are short lived and are difficult to detect directly (107). During the measurements, some necessary procedures should be taken care of. For example, tissue collection and preparation must be carried out in buffers supplemented with antioxidants, such as diethylenetriaminepentaacetic acid (DTPA) and butylated hydroxytoluene (BHT). The use of antioxidant buffers may be especially critical for the detection of trace amounts of oxidized

products formed during aging or under oxidative stress. In addition, all buffers should be treated with nitrogen before use (1011).

### 1.7.1 Determination of Methionine Sulfoxide Reduction and Methionine Oxidation

The first MetSO reduction assay was developed by Brot and collaborators (1012) on the basis of the observation that MSR-A can reduce *N*-acetyl-MetSO to *N*-acetyl-Met. These authors used an isotope-labeled *N*-acetyl[$^3$H] MetSO as substrate and employed ethyl acetate extraction to assay the resulting radioactive *N*-acetyl-Met. However, disadvantages of this method include the use of radioactivity, incomplete extraction of the reaction product, and its contamination with the substrate. The authors also described an assay for detection of free MetSO reduction activity (1012). In this method, [$^3$H]MetSO is reduced by MSRs the reaction mixture fractionated by thin layer chromatography, and the Met spot visualized by ninhydrin treatment and extracted for quantification of radioactivity.

Another method that is commonly used employs dabsylated MetSO as the substrate (1013), and the product, dabsylated Met, is detected in an HPLC procedure by monitoring the absorbance at 436 nm. This method has a high accuracy and sensitivity, but it requires an HPLC system and significant expertise in the procedure and substrate preparation is time consuming. An enzyme-coupled method, which relies on the detection of the absorbance change of NADPH at 340 nm, is also used (1014). In this method, the reduced state of MSR-A or MSR-B is regenerated during the reaction with Trx, which in turn is reduced by NADPH-dependent Trx reductase. The detection limit of this assay is lower than that of the HPLC assay; moreover, it can be used with both free and dabsylated MetSO forms of the substrate.

Le et al. (1015) computationally identified natural Met-rich proteins (MRPs) and characterized three such proteins containing 21–33% Met residues. Oxidation of multiple Met residues in MRPs with $H_2O_2$ and reduction of Met sulfoxides with MSR-A/MSR-B dramatically influenced the mobility of these proteins on polyacrylamide gels and could be monitored by simple SDS-PAGE. They further prepared antibodies enriched for reduced and Met sulfoxide forms of these proteins and used them to monitor Met oxidation and reduction by immunoblot assays. They hypothesized that reduced and oxidized MRPs will have significant structural differences and be amenable to generation of antibodies with a higher affinity for MetSO. Interestingly, upon oxidation with $H_2O_2$, MRPs migrated more slowly on SDS-PAGE gels, and this shift was attributed to Met oxidation. Mass spectrometry analyses also showed the mass shifts characteristic of Met oxidation. Thus, the altered mobility of oxidized MRPs of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels was due to oxidation of Met residues, and this property could be used for gel-based analyses of their Met redox state. Furthermore, they prepared a selenomethionine form of an MRP and found that selenomethionine selenoxide residues

can be efficiently reduced nonenzymatically by GSH and other thiol compounds.

### 1.7.2 Determination of Protein Glycation and Adducts

Early studies assessed glycation with thiobarbituric acid (TBA), which is known to nonspecifically cross-react with a number of enzymatically bound sugars and possibly lysine oxidase-derived aldehyde and ketoamine cross-links of collagen (626, 1016). Further studies measuring glycation products by boronate affinity chromatography have shown no increase with age in skin (1017) or basement membrane glomeruli (1018). More recent studies measuring the acid-hydrolyzed products of glycation as furosine using C18 reverse-phase HPLC have shown either a fairly large increase in aorta with substantial variation occurring especially after 50 years (1019), or slight increases in tendon and aorta (1020). A technique based upon measuring furosine levels by selected ion monitoring and gas chromatography–mass spectrometry (GC-MS) has shown a very modest increase occurring between the ages of 20 and 85 years in human skin (631).

Glycation can be also measured by sodium [$^3$H] borohydride reduction method or using anti-AGE monoclonal antibodies, for example, by using the 6D12 antibody. Ikeda et al. (1021) identified the CML modification as the 6D12 target. Quantitative enzyme-linked immunosorbent assays (ELISAs) for AGEs were developed, often using CML as the epitope (625, 1021, 1022). Others were also using polyclonal antibodies as the primary antibody in the ELISA (1023), whereas Nakamura et al. (1024) used this antibody to demonstrate an inhibited AGE formation by OPB-9195. Besides ELISAs, immunohistochemistry methods are often used to measure AGE modifications (1025). ELISA assays were introduced for the measurement of serum AGE-modified proteins (1026) or erythrocytes containing AGEs (1027). Several specific antibodies, for example, against pyrraline or pentosidine, were developed (1028–1029). These can be used also in clinical investigations as in diabetic patients (1028), or also for the establishments of ELISAs (1029). Haberland et al. (1030) used antibodies to MDA-modified proteins and reported such proteins in atherosclerotic plaques. It was also shown that both MDA-Lys and HNE-Lys epitopes are present in atherosclerotic plaques (1031, 1032), are enriched in senile plaques in the brain(1033, 1034), and in liver and plasma proteins if exposed to oxidative stress (1035). Bucala et al. (1036) detected both lipid-linked and protein-bound AGEs on human LDL by ELISA. It was proposed that the so-called AGE lipids are the result of carboxymethylation of phosphatidylethanolamine (1037). Therefore, Requena et al. (1038) confirmed the presence of N-(carboxymethyl)-phosphatidylethanolamine and N-(carboxymethyl)-phosphatidylserine. Interestingly, N-(carboxymethyl)ethanolamine is able to cross-react with CML-specific antibodies, giving false-positive signals for protein modification (1038). Therefore, MDA adducts to

phosphatidylethanolamine have been detected in red cell membranes (1039), so that in reality, "MDA protein" epitopes in plaques might be actually be lipid derivatives.

Often the antibodies used in AGE protein detection might be not highly specific, so similarities in the core structures of furanyl-furoyl-imidazole (FFI) (1040) and of the imidazolium cross-links, GOLD and MOLD (1041), suggest possible cross-reactions. In contrast, similarities in the pyridinium ring structures of pentosidine (630) and cross-lines (1042) may also yield cross-reacting antibodies. Proof of formation of one or the other AGEs has to be done by chemical methods (1043). Giardino et al. (1044) recently detected an increase in endothelial AGE-modified proteins if cells were exposed to a high glucose medium. A more or less specific modification of the proteins was proposed.

### 1.7.3 Analysis of Isoaspartate Formation

The determination of isoaspartate formation involves the use of protein-L-isoaspartyl methyltransferase (PIMT). PIMT catalyzes methyl transfer reactions using methyl-S-adenosyl-L-methionine (SAM) as the methyl donor. Because PIMT is highly specific for protein isoaspartate residues, the use of tritiated SAM, where the methyl group is radiolabeled [$^3$H]methyl-S-adenosyl-L-methionine, provides a quantitative method for the determination of isoaspartate residues formed in proteins/peptides. To identify which proteins in a tissue sample or a protein mixture contain isoaspartate residues, the protein sample, incubated with [$^3$H]SAM and PIMT, can be further analyzed by SDS-PAGE after mixing with acid SDS-PAGE sample buffer. Protein bands can then be excised from the gel and solubilized in 30% hydrogen peroxide; however, for the measurement of isoaspartate formation, acid SDS-PAGE (pH 2.4), rather than the Laemmli gel system (pH 8.3), should be performed. This is necessary, as the high pH running buffer will hydrolyze the base-labile isoaspartate methyl esters, leading to the release of [$^3$H]methanol into the running buffer (1011).

### 1.7.4 Measurement of Fragmentation

The measurement of oxidative protein fragmentation, which, unlike proteolysis, does not always involve the formation of new N-termini, is not straightforward. In most cases, only qualitative measurements have been made. Quantification of fragmentation of isolated proteins by oxidants using SDS-PAGE under reducing conditions (555) is difficult, as the fragments are often small and difficult to retain (during electrophoresis and/or staining), and individual fragments may be stained differentially depending on their composi-

tion and degree of oxidation (920). There have been attempts to correlate fragmentation with terminal amino group generation. When amino groups were measured in proteins exposed to $^{\bullet}OH$ after unfolding induced by guanidine hydrochloride, modest increments in total amino groups were observed after low doses, with losses at higher doses (1045). Similarly, exposure of lysozyme to $Cu(II)/H_2O_2$ resulted in an initial loss of ε-amino groups of lysine and a subsequent increase in soluble amino groups; if the lysine groups were blocked prior to oxidation, only modest increases were observed. Thus, until precise measurements of the competing pathways are obtained, the release of trichloroacetic acid-soluble amino groups is an unreliable index of fragmentation in *in vitro* systems. Accurate quantification of released materials requires knowledge of the exact amounts of protein present. Determination of Kjeldahl nitrogen or total amino acids after hydrolysis provides such data, although this can be difficult to achieve with oxidized fragments. A more reliable method of measuring fragmentation is to use proteins radioactively labeled in either main- or side-chain sites. These can be produced by biosynthesis, reductive methylation (920), or iodination. With the last of these, routinely used in cellular metabolism studies, the free iodide generated can be problematic and needs to be removed by treatment with silver nitrate.

### 1.7.5 Measurement of Tyrosine Oxidation

To date, the detection of dityrosine formation as a measure of protein oxidation has been carried out by HPLC (1046, 1047), mass spectrometry (1048), and time-resolved fluorescence studies (1049). Van der Vlies et al. (1050) presented a highly sensitive method for the detection of tyrosyl radical formation in cells. The method is based on the fluorescein-labeled tyrosine analogue, tyramine, which upon oxidation may couple to proteins carrying a tyrosyl radical. Coupling of the probe (denoted TyrFluo) to standard proteins could be induced by generating ROS with horseradish peroxidase/hydrogen peroxide, SIN-1, or with peroxides (cumene or hydrogen peroxide) in combination with a transition metal. TyrFluo added to rat fibroblasts remained outside the cell, whereas the acetylated form (acetylTyrFluo) was membrane permeable and accumulated in the cell. Exposure of the cells to oxidative stress in the presence of either TyrFluo or acetylTyrFluo gave a cellular labeling characteristic for each probe. The presence of fluorescein means that the tyrosylated proteins can be visualized in cells by fluorescence microscopy and on a blot by immunodetection using an antifluorescein antibody. To determine the sensitivity for tyrosylation, a set of standard proteins (phosphorylase B, BSA, ovalbumin, carbonic anhydrase II, trypsin inhibitor) was exposed to various oxidative conditions ($H_2O_2/Fe^{2+}$, SIN-1, $HRP/H_2O_2$, and CumOOH/hemin) in the presence of Tyr-Fluo. Under each of these conditions, the proteins were shown to become labeled, indicating that both tyramine and tyrosine residues

are converted into tyrosyl radicals and available to form an o,o′-dityrosine bond.

### 1.7.6 Protein Carbonyl Measurement

Protein carbonyls are the most widely measured biomarkers of protein oxidation, as they are, in general, stable products and formed relatively early during oxidative stress. Thus, the quantification of protein carbonyls as an indicator of ROS-mediated protein modification is a useful tool in biochemical stress research (1051).

At present, the most widely used assay for protein carbonyl detection involves the derivatization of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH). Derivatization with DNPH leads to the formation of a stable protein-conjugated dinitrophenylhydrazone product, which has a peak absorbance of nearly 360 nm. Therefore, DNPH provides a basic agent for the quantification of protein carbonyl content in purified proteins as well as in protein mixtures. This hydrazone derivate can be quantified by different methods, including spectrophotometric (1052), one-dimensional or two-dimensional electrophoresis followed by immunoblotting (1053), immunohistochemistry (1054, 1055), HPLC (1056), and ELISA (1057).

The spectrophotometric method is generally not recommended because of practical problems. The most important disadvantage of the spectrophotometric assay is its high protein requirement, sometimes more than the available in research or clinical samples (1052). Quantification of protein carbonyls by spectrophotometry following DNPH modification is sometimes not feasible (with the proteins that contain high amounts of chromophore that absorbs at 360 nm such as hemoglobin, myoglobin, or retinoids) and in these cases, the reaction of carbonyls with tritiated sodium borohydride provides an alternative method for quantitative measurement. Tritiated sodium borohydride transforms protein carbonyls to protein-bound ethanol groups, and tritium is simultaneously incorporated into these proteins (1011). Consequently, the protein is not reacting with DNPH and the real protein carbonyl value can be determined by subtraction of the absorbance value obtained following tritium incorporation from the absorbance value without tritium incorporation.

A widely used assay is the protein carbonyl ELISA method with different modifications between laboratories. This method can be applied to cells, tissue, and plasma. Its advantages are reliability and sensitivity and, further, the assay can be applied to both experimental studies and clinical samples (1058–1060). Free DNPH and nonprotein constituents are easily washed away during ELISA performance and give minimal interference. This results in a higher sensitivity and accuracy at lower protein carbonyl concentrations compared to the spectrophotometric DNPH assay. The first protocol for quantifying protein carbonyls by ELISA was developed by Buss et al. (1057). Further

protocols described included some modifications as in Sitte et al. (1058) and in Voss et al. (1059). In this method, samples can be used fresh or stored at –80°C, but before starting with the determination of carbonyls, the protein concentration of the samples should be determined. The commercially available Bradford reagent can be used for determination of protein content. All samples and standards should be adjusted to 4 mg/mL or the lowest protein concentration in order to have the same protein concentration (minimum 1 mg/mL). If the protein concentration is very low, then the samples should be concentrated or an alternative method should be used (1061). Oxidized BSA should be prepared in advance and will be used as a standard. A serial dilution of oxidized and reduced BSA should be prepared for the standard curve using the same protein concentration as in the sample. A minimum six-point standard curve of oxidized BSA with reduced BSA should be included in each multiwell plate. DNPH reagent in PBS without protein should be used as blank for the calculation of samples. DNPH reacts with the free carbonyl groups of the absorbed proteins, leading to adduction of the dinitrophenyl (DNP) group to the carbonyl group. A primary antibody to DNP and a secondary antibody, which is antirabbit IgG POD, are used. Peroxidase (POD) in the secondary antibody reacts with substrates o-phenylenediamine and 3,3′,5,5′-tetramethylbenzidine dihydrochloride together with hydrogen peroxide. The latter is used in the method for minimal yield of proteins instead of o-phenylenediamine because of its higher sensitivity. Protein carbonyl measurement procedures used in different laboratories are often not precisely specified in the published papers. This point is of crucial importance when comparing data from different working groups because there are considerable variations in the basal levels of protein carbonyls in certain literature sources, depending on how the carbonyl assay is performed (1051).

If protein concentration is a limiting factor, or when the studied proteins such as heme-containing proteins have absorption maxima around 360 nm, protein carbonyls cannot be determined by the use of DNPH. In such cases, tritiated sodium borohydride [$^3$H]$NaBH_4$, which is able to convert protein carbonyls into protein-bound ethanol groups, can be used. Because of the introduction of tritium into the oxidized proteins, the method provides a quantitative measurement of protein carbonyl content. Protein samples derivatized with tritiated sodium borohydride can also be separated by SDS-PAGE. The target protein band on the gel can then be excised and dissolved in 30% hydrogen peroxide, and incorporated radioactivity measured by the use of a scintillation counter (1011).

Another alternative method is the derivatization of protein carbonyls with biotin-hydrazide. This protocol provides an additional method by which protein carbonyls are derivatized with biotin hydrazide for the analysis of carbonylated proteins. The biotin-tagged proteins can then be resolved by 2D gel electrophoresis and detected on immunoblots probed with streptavidin. If desired, proteins showing an increased level of carbonylation can be identified by mass spectrometric techniques (1011).

Yan et al. in 1998 (1062) developed a 2D SDS-PAGE method for protein carbonyls. In this method, together with microsequencing, there are four specific steps which are SDS-PAGE of protein samples without DNPH treatment, isoelectric focusing (IEF) of the stained polypeptides corresponding to those exhibiting positive carbonyl accumulation, concentration of the protein excised from a stained IEF gel by SDS-PAGE, and microsequencing. After electrotransfer of the protein onto Immobilon-P membrane, the protein is subjected to automated N-terminal microsequencing, gas-phase sequencer (1063). In this method (1062), instead of using a 2D system for the identification of the protein, another simple method is preferred. This is because matching and excision with all controls and samples being run on separate SDS-PAGEs following IEF are virtually impossible if 2D gels are used. Another advantage of this method is that the oxidized proteins can be concentrated because several IEF bands can be loaded in one well of SDS-PAGE. This is unlike 2D gels, where loading capacity of both sample volume and protein amount is limited (1064).

Mirzaei and Regnier (1065) reported a method for the isolation of oxidized peptides, which involves derivatization of oxidized proteins with Girard P reagent (GRP; 1-(2-hydrazino-2-oxoethyl)pyridinium chloride), following proteolysis enrichment of the derivatized peptide using strong cation exchange chromatography, and identification of oxidation sites using tandem mass spectrometry (MS/MS). Derivatization of aldehydes and ketones in oxidized proteins is achieved by reacting protein carbonyls with the hydrazide of GRP. Although GRP was originally developed to derivatize and solubilize insoluble steroids, it readily derivatizes oxidized proteins. An attractive feature of GRP is that it carries both a hydrazide and quaternary amine group. The hydrazide group reacts readily with carbonyls, whereas the quaternary amine adds positive charge to oxidized proteins and peptides. The resulting hydrazone bond is reduced by sodium cyanoborohydride to further stabilize the labeling.

Protein carbonyl groups also react with hydrazine to form a Schiff base, which can be reduced to stable secondary amines that are easily quantified. Another way is through derivatization with a fluorophore such as fluorescamine. The resulting secondary amine is fluorescent and has high molar absorptivity at 489 nm (1066). Protein carbonyl groups have also been labeled with digoxigenin hydrazide and detected by dot blotting with an antidigoxigenin antibody (1067).

A recent method developed for identification of oxidized proteins utilizes biotin hydrazide as a labeling reagent to react with protein carbonyls and provide an affinity tag for the chromatographic isolation of oxidized proteins (1068). After avidin affinity capture of oxidized proteins, they were further fractionated by reversed-phase chromatography. Fractions from the reversed-phase column were then tryptic digested and the proteolytic digests either analyzed directly by electrospray ionization–mass spectrometry (ESI-MS) or after further fractionation with reversed-phase chromatography. Even though

this method allows comprehensive analysis of oxidized proteins by identification of all oxidized and nonoxidized peptides pooled in the same fraction, it has its own drawbacks. Identification of all peptides of oxidized proteins to identify the site of carbonylation is time consuming and labor intensive. The biotin hydrazide tag can also fragment and interfere with the fragmentation pattern of the peptide (1065). As a result, biotinylated peptides are either missed or search engines can have difficulty assigning the sequence. Moreover, some nonspecific binding of the hydrazide to the protein (1069) and the endogenous biotinylation within cells can also be an issue (1070).

Immunologically, besides ELISA methods, DNPH-derivatized protein products can be separated by molecular weight using one-dimensional electrophoresis (1-DE), blotted to a support matrix (e.g., PVDF), and visualized by immunostaining with antibodies that recognize the DNP portion of the hydrazone. One-dimensional electrophoresis (1-DE) can resolve many protein mixtures, but complex biological samples require high-resolution 2-DE for critical protein identification. Although the DNP/1-DE method for detection of protein carbonyls is widely used (1071, 1072), the modification of this protocol for high-resolution 2-DE has been more challenging. If derivatization of protein samples is performed prior to 2-DE, some problems may occur. These are classified as below: the salts and detergents used in the derivatization process must be removed prior to IEF, the extreme pH conditions required for derivatization of the carbonyls can alter the isoelectrophoretic mobility of proteins, and 2-DE is a multistep process during which the additional steps amplify variability and decrease reproducibility. Consequently, protein samples subjected to DNP derivatization prior to IEF often yield varied and irreproducible 2-DE protein fingerprint profiles when compared with the same samples that were not DNP derivatized. Thus, resultant DNP-derivatized protein fingerprints cannot be directly compared to the growing number of annotated 2-DE protein databases available such as ExPASy and SWISS-2D PAGE. Therefore, methods that employ DNP derivatization after 2-DE separation and allow comparisons of protein fingerprints with annotated databases would offer major advantages over methods that use DNP-derivatization prior to 2-DE. Since prederivatization alters the electrophoretic properties of a given protein, Conrad et al. (1073) developed a new method for postelectrophoretic derivatization of proteins immobilized on the PVDF membrane. PVDF membranes are preferred for immunoblotting. However, some researchers suggested that polyacrylamide gels and nitrocellulose membranes both exhibited strong yellow backgrounds after exposure to DNPH, making quantitative evaluation of proteins impractical. This background did not reach acceptable low levels even after extensive washing. The best results were obtained with PVDF membranes, but the background was still considered unacceptable. Solvents which removed the yellow stain from the PVDF membrane also tended to wash off the protein. Therefore, efforts were directed to separation and identification of prederivatized proteins (1074).

Of those stains for detection of protein on membrane blots (1075, 1076), most worked best with PVDF membranes, whereas nitrocellulose membranes resulted in high backgrounds. Since SDS and guanidine hydrochloride are not compatible with IEF, it is necessary to solubilize the DNP-derivatized protein in a buffer containing a nonionic denaturant such as urea. Although SDS and urea do not themselves interfere with DNPH derivatization of protein carbonyls, trace impurities may (1074). Losses of membrane proteins during 2D-PAGE due to their adsorption on IPG matrices have also been reported (1077, 1078).

A specially designed oxidation-dependent carbonyl-specific element-coded affinity mass tag (O-ECAT), AOD, ((S)-2-(4-(2-aminooxy)-acetamido)-benzyl)-1,4,7,10-tetraazacyclododecane-N,N′,N″,N‴-tetraacetic acid, is used to covalently tag the residues of a protein oxidized to aldehyde or keto end products. O-ECAT can be loaded with a variety of metals, which yields the ability to generate mass pairs and multiplex multiple samples. The O-ECAT moiety also serves as a handle for identification, quantitation, and affinity purification. After proteolysis, the AOD-tagged peptides are affinity purified and analyzed by nanoLC FTICR MS (nanoliquid chromatography–Fourier transform ion cyclotron resonance–mass spectrometry), which provides high specificity in extracting coeluting AOD mass pairs with a unique mass difference and allows relative quantitation based on isotopic ratios. Using this methodology, Lee et al. (1079) have quantified and mapped the surface oxidation sites on a model protein, recombinant human serum albumin (rHSA), in its native form (as purchased) and after FeEDTA oxidation both at the protein and amino acid levels. AOD has two key elements: (i) an aminooxy group that can form a covalent bond with aldehydes or ketones and (ii) a DOTA (1,4,7,10-tetraazacyclododecane-N,N′,N″,N‴-tetraacetic acid) metal-chelator moiety. AOD is water soluble and can be loaded with a wide choice of monoisotopic rare earths to generate a series of mass tags with large mass defects (1080) and nearly identical chromatographic behavior.

Chaudhuri et al. (1081) have adapted a fluorescence-based approach using fluorescein-5-thiosemicarbazide (FTC) to quantify the global protein carbonyls as well as the carbonyl levels on individual proteins in the proteome. Protein carbonyls generated *in vitro* were quantified by labeling the oxidized proteins with FTC followed by separating the FTC-labeled protein from free probe by gel electrophoresis. The reaction of FTC with protein carbonyls was found to be specific for carbonyl groups.

### 1.7.7 Radioactive Labeling Protocols for Proteolysis and Aggregation Measurements

Early work (1082) used nonproteogenic amino acids, which are artificially used in protein synthesis. However, using this approach, malfolded proteins are always synthetized. Therefore, the most convenient way of measuring proteolysis of modified proteins in functionally intact cells is accepted to be the use

of techniques based on the radioactive quantification of amino acids using liquid scintillation counting (1083). This method has been used in many studies to measure the changes in protein degradation (42, 934, 1084, 1085) and protein aggregate formation (1086, 1087) after various treatments.

Liquid scintillation counting is an analytical technique for measuring radiation from β-emitting nuclides such as $^{14}C$, $^{3}H$, $^{35}S$, $^{32}P$, and $^{63}Ni$, and is the most frequently used method to detect radioactivity in biological samples labeled with isotopes (1088, 1089). Samples are dissolved or suspended in a cocktail containing an aromatic solvent and small amounts of other additives known as fluors. Sample preparation is critical for proper counting because the energy transfer process necessitates close contact between the sample and the fluor in the counting solution. β-Irradiation emitted from the sample transfers the energy to the solvent molecules, which in turn transfer their energy to the fluors; the excited fluor molecules dissipate the energy by emitting light. In this way, each β-emission results in a pulse of light. The primary and most extensively used scintillation cocktails contain 2,5-diphenyloxazole, toluene, xylene, and dioxane. During measurement, the samples are placed in small transparent or translucent (glass or plastic) vials that are loaded into a liquid scintillation counter. The counter has two photomultiplier tubes connected in a coincidence circuit. The coincidence circuit ensures that genuine light pulses, which reach both photomultiplier tubes, are counted, whereas spurious pulses (due to noise, for example), which would affect only one of the tubes, are ignored (1090).

Protein oxidation/fragmentation or proteolysis determinations include either the degradation of metabolically radiolabeled cellular proteins or the degradation of chemically radiolabeled proteins or peptides (1085). Additionally, with the metabolic labeling of intracellular proteins, further information can also be obtained, including the formation of insoluble protein complexes due to aggregation of unfolded, denatured proteins (1087), the turnover of single proteins, and more. $^{125}I$, $^{3}H$, or $^{14}C$-containing groups are attached to the proteins. However, because $^{125}I$ irradiates high-energy irradiation, the use of this isotope is not recommended for testing the degradation of oxidized proteins (1091–1092). Hence, $^{3}H$ or $^{14}C$ labeling is used more often. Radioactively labeled ($^{3}H$ or $^{14}C$) formaldehyde is used and added to amino groups by reductive alkylation. Sodium borohydride or sodium cyanoborohydride is used as a reducing agent for Schiff bases (1093, 1094).

Another way is the metabolic labeling of proteins in intact cells by incorporation of exogenously added radioactive amino acids. Metabolic labeling of cellular proteins is achieved by placing cells in a nutritional medium containing all components necessary for the growth of the cells in culture, except for one amino acid, which is substituted by its radiolabeled form. The radiolabeled amino acids are transported across the plasma membrane by carrier-mediated systems and, once in the cytosol, are loaded onto tRNA molecules before being incorporated into newly synthesized proteins (1095). Because metabolic labeling techniques use the metabolic machinery of the cell to incorporate

radiolabeled amino acids, there are limitations on the type of radiolabeled amino acids that can be employed. The list of potential precursors is restricted to L-amino acids normally found in proteins, in which one or more atoms are substituted by a radioisotope (1095) (Table 1.1).

[$^{35}$S]Methionine/Cys is the most frequently used radiolabeled amino acid because of its high specific activity. For proteins that contain little or no methionine/Cys, other amino acids labeled with $^{3}$H and $^{14}$C can also be obtained (1091). [$^{3}$H]Leucine, is a good alternative to $^{35}$S-labeled amino acids. But several problems can arise when using certain $^{3}$H-labeled amino acids, owing to their participation in metabolic pathways. When choosing the labeled amino acids, specific activities and half-lives have to be considered. The incorporation of radioactivity into the intracellular protein pool can be used as a measure of protein synthesis. Here it is recommended that rather short labeling times should be used because otherwise multiple factors might influence the measurement. The incorporated radioactivity is determined as either the difference between the added radioactivity and the removed radioactivity from the tissue culture dish or the amount of resolved radioactivity after TCA precipitation of proteins. Both methods may have problems based on the precipitation of small amounts and difficulties in a 100% controlled solution of the precipitate or on the potentially large errors in volume measurements and calculations, and care should be taken to manipulate the cell number used or the time of incorporation to ensure that a sufficient amount of radioactivity is incorporated (1096).

To determine the proteolysis rate, the cell-labeling medium has to be removed and the cells have to be washed. Afterward, normal tissue culture medium (containing a high concentration of the amino acids used for labeling, but in a nonradioactive form) has to be added. This is required to ensure that after a protein is degraded, and the radioactive amino acid is liberated, it is not used for protein synthesis again. For the measurement of proteolysis, two methods are possible. One is based on the assumption that the exchange of amino acids between the cytosol and the extracellular medium is very quick (at least much faster than proteolysis processes, which often take hours). If this is true, the measurement of TCA-soluble radioactivity in the medium of the tissue culture is sufficient. (Note: This assumption has to be tested for each cell line used and for each amino acid used for labeling.) If this assumption is not true (or for testing whether it can be assumed), proteolytic degradation of metabolically labeled cellular proteins should be quantified either by precipitating the cellular proteins, resolving them, and counting the liquid scintillation or by using the whole content (medium plus cells) of the tissue culture well, precipitating it with TCA, and measuring the released radioactivity. To do so, proteins will be precipitated in ice-cold TCA with a final concentration of 10% and centrifuged at 14,000 *g* for 10 min (42), followed by scintillation counting of supernatant. Performing such a procedure allows counting of all amino acids and peptides with a molecular weight below 5 kDa.

**TABLE 1.1 The Use of Radiolabeled Amino Acids to Label Proteins**[a]

| Amino Acid[b] | Frequency (%)[c] | Radioisotope | Specific Activity | Organ or Tissue[d] | Cells[d] |
|---|---|---|---|---|---|
| Leucine | 10.4 | $^{3}H$ | 5–190 | Brain, heart, | Ovary cells, neurons, pneumocytes |
| | | $^{14}C$ | 0.22–0.3 | Liver, muscle | Fat cells |
| Lysine | 7.0 | $^{3}H$ | 40–110 | Kidney, brain, retina, thymus | Ovary cells |
| | | $^{14}C$ | 0.22–0.26 | Kidney, brain, liver, spleen | Nerve cells, blood cells |
| Valine | 6.2 | $^{3}H$ | 10–65 | Liver, brain, heart, jejunum | Astroglial cells, hepatoma cells |
| | | $^{14}C$ | 0.18–0.22 | Liver, brain, kidney, skeletal muscle | Blastocysts |
| Threonine | 5.6 | $^{3}H$ | 5–25 | Bronchial submucosal gland, colonic mucosa | |
| Histidine | 2.5 | $^{3}H$ | 30–70 | Brain, intestine, nerve tissue | |
| Isoleucine | 2.9 | $^{3}H$ | 30–140 | Brain, liver | Erythrocytes |
| | | $^{14}C$ | 0.22–0.26 | Brain, liver, white muscle | Fibroblasts |
| Cys | 3.4 | $^{35}S$ | >800 | Liver, brain, pancreas, kidney, heart | Leukocytes, fibroblasts, K562 cells, epithelial cells, and so on |
| Methionine | 1.8 | $^{35}S$ | >800 | Brain, epididymis | Lymphoblasts, reticulocytes, chromaffin cells, fibroblasts, ganglion cells, and so on |

[a]According to Catalgol and Grune, Free Radic. Biol. Med. 46: 8–13, 2009 and Bonifacino, Curr. Protoc. Mol. Biol. 44: 1–10, 1998.
[b]All amino acids are in the L-configuration.
[c]Frequency of amino acid residues in proteins.
[d]Organs, tissues, and cells that were used for radiolabeling with the corresponding amino acid.

The solubility of proteins in a detergent cocktail is used as a quantitative indicator of protein aggregation (1087). After metabolic labeling, cells are often treated with oxidative stress, and the formation of protein aggregates is the readout. Proteins, which are released by sonication of the cell pellets, are solubilized in a detergent cocktail. Detergent-soluble proteins are counted to determine the decrease in the solubility and to quantify the protein aggregates; detergent-insoluble proteins are counted after solubilization in NaOH (1086).

### 1.7.8 Standard Chromatographic Methods for the Measurement of Protein Modifications

Oxidation of the C2 position of the imidazole ring of histidine converts the residue to 2-oxohistidine. Lewisch and Levine described procedures for stabilizing 2-oxohistidine which allow its quantification by routine methods of amino acid analysis. These include classical ion exchange chromatography with postcolumn derivatization by o-phthaldialdehyde, reverse-phase chromatography with precolumn derivatization by o-phthaldialdehyde, and reverse-phase chromatography with precolumn derivatization by 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. Using these techniques, a previously unidentified amino acid, which appears during the oxidative inactivation of GS, was shown to be 2-oxohistidine. One picomole of 2-oxohistidine was readily detected in a protein hydrolysate containing 1700 pmol total amino acids (867).

Early attempts to detect 3-NY used a combination of high-performance liquid chromatography with ultraviolet detection (HPLC-UV) and immunochemical methods. Although both approaches met with some success, both were inherently limited. UV detection is sufficiently sensitive for determination of 3-NY *in vitro* but generally not sensitive enough for the routine quantitation of 3-NY *in vivo*. Salman-Tabcheh and colleagues (1097) measured free 3-NY in human plasma using reverse-phase HPLC with UV detection; however, few similar studies have been reported. An HPLC–electrochemical detection (ECD) array technique was used to measure 3-NY and other tyrosine oxidation products in cell culture (1098), human brain tissue (1099), and cerebral microvessels (1100). It is critical for an ECD experiment that the mobile phase has sufficiently high ionic strength to facilitate electrochemical reactions at the electrode surface. For this reason, lithium acetate or lithium triphosphate (30 mM or higher) is recommended for use in the mobile phase in combination with an acetate or citrate buffer with a pH less than 5.0. It should be noted that various strategies for ECD of 3-NY have been reported. These typically rely on precolumn chemical reduction of the analyte to 3-aminotyrosine by treatment with strong reducing agents such as sodium hydrosulfite (1101). Aminotyrosine is more easily oxidized than 3-NY; hence, it is more readily detected than the 3-NY precursor.

Chemical determinations of six protein-bound oxidation products, DOPA, o-tyrosine, m-tyrosine, dityrosine, hydroxyleucine, and hydroxyvaline, have

been achieved by HPLC measurement. Two steps of HPLC, including fractionation on an LC-NH2 column with a Pelliguard column followed by ODS column, were needed for the quantification of 3-hydroxyvaline and the 5-hydroxyleucines. For detection of DOPA, m-tyrosine, o-tyrosine and dityrosine, and hydrolysate were chromatographed on an ODS column with a Pelliguard guard column (193, 1102).

Several different methods have been developed to study protein–protein interactions. A common feature of oxidative stress-induced protein aggregates is that they should be of higher molecular weight and size than the individual protein components of which they are composed. Size exclusion chromatography (SEC) was used in this work for differentiation on the basis of size in recognizing and isolating protein complexes. Considering the fact that many nonspecific protein–protein interactions are often hydrophobic, nondenaturing conditions should be used in the separation method selected to avoid disturbing the interactions that hold the aggregate together. SEC has all the specifications required for size-based fractionation of complex protein mixtures without denaturation. Aggregates fractionated by SEC can also be analyzed by a wide variety of standard proteomics platforms that will detect and identify protein components (1103).

### 1.7.9 Liquid Chromatography Techniques Supported by Mass Spectrometry

The development of high-resolution separation and mass spectrometry instrumentation has opened the door for the identification of functionally important posttranslational protein modifications relevant to disease and the aging process.

The bulk of studies showed the decrease in histidine levels, but the increase in 2-oxohistidine levels could not have been proved analytically (560). These findings raised the question whether, specifically, 2-oxo-histidine is actually formed during protein oxidation *in vivo* or whether it only escapes detection due to a potentially rapid degradation of the product itself or of proteins containing 2-oxo-histidine. New data on the MCO of the PerR transcription factor in Bacillus subtilis provide a partial answer to this question (1104). ESI-MS trials show that 2-oxo-histidine can be formed *in vivo* and suggest that 2-oxo-histidine can be sufficiently stable for chemical analysis.

Brock et al. (1105) described the development of a liquid chromatography–tandem mass spectrometry (LC-MS/MS) for quantification of methionine oxidation at specific sites in the model protein ribonuclease (RNase) during glycoxidation or lipoxidation by glucose or arachidonate, respectively. Control, glycoxidized, and lipoxidized RNase were digested with trypsin and analyzed by reversed-phase HPLC, electrospray ionization triple quadrupole mass spectrometry. Percent oxidation of Met in peptides was determined as the sum of the peak areas of the charged forms of the MetSO peptides, divided by the sum of the peak areas of the nonoxidized (Met) and oxidized (MetSO)

peptides. Sequencing of peptides was performed on the QTOF, with the quadrupole set on mass of the peptide of interest and the TOF scanning for daughter ions between 50 and 3000 atomic mass units, using collision energies of 30–40 eV.

Because of the difficulties associated with their insolubility and handling, skin keratin proteins were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), liquid chromatography electrospray ionization mass spectrometry (LC-ESI/MS), and MS/MS. Peptide mass fingerprinting (PMF) analyses have identified keratins K1 and K10 as the major proteins of human skin. Met(259), Met(262), Met(296), and Met(469), located in the α-helical rod domain of K1, were found to be the most susceptible sites to oxidation, induced by hydrogen peroxide *in vitro* and *in vivo* (1106).

### 1.7.10 GC/MS

Oxidation of arginine and proline results in γ-glutamyl semialdehyde, which can be chemically reduced and forms 5-hydroxy-2-amino valeric acid (HAVA), which can be measured after modification by GC/MS selected ion monitoring (1107). As glutamic semialdehyde is a product of oxidation of arginine and proline, so is aminoadipic semialdehyde (AAS) a product of oxidation of lysine. Sensitive GC/MS-based analytical methods allow the quantitation of both in a variety of biological samples, including tissue protein, cell cultures, and lipoproteins (1108).

Trace amounts of dityrosine residues formed in proteins due to oxidative damage are first released by acid hydrolysis. The released dityrosine residues are then analyzed by mass spectrometry following derivatization with heptafluorobutyric anhydride/ethyl acetate. For the preparation of standards, dityrosine can be formed by horseradish peroxidase-catalyzed oxidation of tyrosine in the presence of hydrogen peroxide. Dityrosine produced in the mixture can then be purified by chromatographic methods. If radiolabeled dityrosine is to be made, radiolabeled tyrosine should be used as a starting material (1011).

### 1.7.11 Analysis of Protein-Bound 3-Nitrotyrosine by a Competitive ELISA Method

3-NY is a useful marker for nitric oxide-mediated tissue injury. The availability of antinitrotyrosine antibodies provides a very sensitive method for the detection of protein-bound nitrotyrosine in tissue samples. In this protocol, nitro-BSA is coated onto ELISA plates, and nitrotyrosines are quantitated by the use of antinitrotyrosine antibodies. Competition is accomplished by adding either a potentially nitrated protein sample or a known amount of nitrotyrosine (in the form of nitro-BSA) as a standard. Each competes with the coated nitrated proteins for antibody binding. The amount of antibody that binds to the coated nitro-BSA is inversely proportional to the amount of nitrated

protein (sample or standard) present in the solution added to the well of the plate (1109).

### 1.7.12 Protein Oxidation Products as Biomarkers in Clinical Science

In this section, we will examine shortly various aspects of protein oxidation, with emphasis on using proteins as markers of oxidative stress in biological samples and clinical science. In clinical studies, biomarkers can be employed to reflect environmental pro-oxidant exposures and dietary antioxidant intake or to serve as a surrogate measure of a disease process. To be truly useful, the biomarker must have some degree of predictive validity. Some proposed biomarkers of oxidative stress might simply prove to be general markers of oxidative damage and relate poorly to disease process and outcome. New research studies must address whether and how biomarkers adequately measure relevant physiological functions or relate to established pathological signs, particularly with regard to their accuracy, precision, and reliability. Such efforts must consider the potential for artifacts produced during sample collection, processing, storage, and instrumental analyses, as well as confounding by the presence of related factors such as the status of facets of the antioxidant defense network that are not under direct study. The validation of biomarkers must include an assessment of the degree of bias in their measurement, especially the characterization of their prevalence and variability within large-scale population studies. An important issue for study is to determine whether specific biomarkers reflect short- or long-term exposure to an antioxidant status or oxidative stress (1110).

During the whole life of a human being, he or she is confronted with oxidative stress, on the one side, from intrinsic origins as the mitochondrial ATP generation is leaking ROS, and on the other side from extrinsic origins like the increasing air combustion in cities by traffic and industrialization (1111, 1112). To evaluate how this oxidative stress is developing during the aging process and how it changes the cellular metabolism, several approches have been used. In the literature, a wide multitude of potential biomarkers are pronounced, including LPO products; protein oxidation products; antioxidative acting enzymes such as SOD, catalase (CAT), Gpx and GR; minerals like selenium, manganese, copper, and zinc; vitamins like vitamin A, C, and E; and GSH, flavonoids, bilirubin, and UA (1113). But none of them could develop to the leading one, which is accepted by the whole scientific community to determine the life expectancy of the individual person, his or her biological age, or his or her age-dependent health status (542, 1114–1117).

There is a bulk of literature using protein-bound carbonyls as a marker for protein oxidation. In a variety of other studies, more specific amino acid modifications such as dityrosine, O-tyrosine or 3-NY, 5-hydroxyl-2-amino valeric acid, or MetSO were used. Changes in overall protein thiol content are also considered as markers. Furthermore, changes in protein structure, often related

to surface hydrophobicity or aggregation, were also used as indicators of oxidation (15).

Generally a set of clinical biomarkers should be used to determine the oxidation status of an individual since one clinical marker is not enough to predict oxidative stress or the life expectancy of a person. To be a reliable and clinically used biomarker for monitoring protein oxidation, the potential parameter has to fulfill some requirements. First of all, the measurement has to be highly reproducible. The value should be stable over the time from the sample drawing until the measurement, and ideally even longer so that it would be possible at later time points to control or add further measurements. Freezing and thawing the sample again should not change the readout of the measurement. As the treatment of ill patients is often dependent on the status of the related biomarker, the measurement should be fast. Short times for a single measurement are also necessary for a high throughput, which makes it possible to screen several patients at the same time. This could then be done by an automated system. By comparing the life span of different species, Ingram et al. (1118) developed a strategy for identifying biomarkers of aging in long-lived species. To find a parameter which is affected only by oxidative stress on the aging process was difficult. For all so far used biomarkers, it is known that they are influenced by either nutrition, lifestyle (smoking, sports, and physical exercise), the individual genetic background, or diseases and medications. Therefore, it is almost impossible to compare the values of biomarkers between patients directly, and this is the reason why large numbers of individuals are necessary to get significant differences in studies monitoring the change of biomarkers with age. As the oxidative markers are not always measured in the same organs and different methods are used by the different laboratories, it is not surprising that the results are often contradictory. A detailed overview over several oxidative amino acid modifications, specific methods to detect them, and their impact on cell metabolism is given in the reviews from Requena et al. (1108) and Stadtman and Levine (825).

The hydroxyl radical converts L-phenylalanine into m-tyrosine and o-tyrosine (61), isomers of the natural amino acid L-tyrosine. m-Tyrosine is also produced by peroxynitrite *in vitro* (1119). Because m-tyrosine is stable to acid hydrolysis and is thought to be absent from normal proteins, it has served as a useful marker for oxidative damage. Thus, elevated levels of m-tyrosine have been detected in aging lens proteins of humans (1120), atherosclerotic tissue of diabetic nonhuman primates (1121), mitochondrial proteins of exercised animals (1122), blood of animals subjected to cardiac ischemia-reperfusion injury (1123), and retinal tissue of diabetic rats (1124). Model system studies indicate that when proteins are oxidized by peroxynitrite, the major product is 3-NY, although low levels of o-tyrosine and m-tyrosine are also detectable (1048, 1125).

A quite new substance which is now investigated as a biomarker is the assessment of branched-chain amino acids (BCAA). BCAA play an important

role in protein and neurotransmitter synthesis. The big disadvantage of this marker is that it is dependent on many clinical states and optimal levels have not been published so far (1126). Besides the analysis of changes in single amino acid levels like the determination of 3-NY, which is a (rather unspecific) marker for peroxynitrite mediated damage (1127), there exist two more general biomarkers of protein oxidation. These are first, the formation of protein-bound carbonyls by the oxidation of the aliphatic side chains of alanine, valine, leucine, and the acid side chain of aspartate, which can be removed from the protein backbone via β-scission; and second, the accumulation of protein aggregates, for example, lipofuscin (1128). It is estimated that almost every third protein in a cell of older animals is dysfunctional as enzyme or structural protein due to oxidative damage (1129). Therefore, the measurement of protein oxidation is a clinically important factor for the prediction of the aging process and age-related diseases. An age-related increase in the protein-bound carbonyl concentration was already reported for tissues like heart, muscle, or brain (15, 823, 1130–1132), as well as in plasma of healthy people (3, 694, 1133, 1134). Traverso et al. (1135) measured lower levels of protein-bound carbonyls in the over 90-year-old people compared with younger. This controversial effect may be explained by the fact that often only very healthy people with generally low oxidation status survive so long and take part in scientific studies. It can be concluded that the measurement of protein-bound carbonyls comprise a cheap, fast, and reliable method to determine oxidative stress on the protein level, as the oxidative damage correlates well with aging even though with the severity of some diseases (1136).

The two major compounds which comprise the group of PCO are γ-glutamic semialdehyde (GGS) and AAS. They are the main oxidation products of proteins after MCO and can reach 55–100% of the total carbonyl content (1108). Daneshvar et al. in 1997 developed an HPLC-based method to detect these two compounds (1137). Roxborough and Young have hypothesized that protein carbamylation (i.e., posttranslational modification of proteins characterized by the binding of cyanate ($CNO^-$) to $NH_2$ groups) could explain the increased incidence of atherosclerosis in patients with renal insufficiency (1138). Even though some experimental studies supporting this hypothesis have been published (1139, 1140), carbamylation has failed to be considered by clinicians as a real etiological factor in the development of specific complications of this disease. By contrast, another nonenzymatic posttranslational modification of proteins, glycoxidation, which requires similar conditions to carbamylation to occur, is widely recognized by the clinical and scientific community for its involvement in the long-term complications of diabetes mellitus (1141). By describing the MPO-induced formation of cyanate as a new and important mechanism of protein carbamylation, the authors contributed to confirm the clinical relevance of carbamylation in human pathophysiology, particularly in atherosclerosis by demonstrating the increased affinity of

carbamylated LDLs for macrophage scavenger A1 receptor. For instance, it was demonstrated that carbamylated collagen was able to stimulate the production of active matrix metalloproteinase-9 by blood monocytes (1142), and that carbamylated collagen as well as carbamylated albumin were able to inhibit respiratory burst of polymorphonuclear neutrophils (1143, 1144).

Modification of structural proteins can also lead to a loss of function and play important roles in several diseases investigated in clinical studies. For example, when the plasma protein fibrinogen is oxidized either by treatment with an iron/ascorbate radical-generating system or with ionizing radiation, it loses its ability to form a solid clot (1145). The degree of clotting inhibition correlates with the extent of carbonyl formation in the protein. Oxidation of synovial fluid immunoglobulins causes aggregation, which may contribute to the etiology of rheumatoid arthritis (1146, 1147), although a correlation between the extent of oxidation of immunoglobulin molecules and severity of disease has not been established. When protease inhibitors such as α-1 antitrypsin become oxidatively modified, severe physiological consequences may ensue. This plasma protein has primary responsibility for inhibiting proteolysis in tissues such as lung and cartilage. Modification of a critical Met residue in α-1 antitrypsin causes a loss of function, which seems to contribute to the tissue destruction seen in emphysema (1148). The primary oxidant responsible for this modification may be HOCl produced by inflammatory neutrophils (1149). Plasma LDL has been demonstrated to undergo several different types of oxidative modification (194, 1030, 1048, 1150–1154). Exposure of LDL to $Cu^{+}$ causes oxidation of the LDL protein, leading to the formation of carbonyl groups (470, 1155, 1156), aggregation (470), and increased cellular uptake by tissue macrophages through the scavenger receptor. Importantly, oxidatively modified LDL has been found in atherosclerotic tissues by several groups, lending strong support for the idea that oxidation of LDL may play a significant role in the etiology of atherosclerosis (1157–1160). Similarly, oxidation of crystalline proteins in the lens of the eye plays a role in cataractogenesis (48, 693, 1161–1163).

Compared to measuring products of LPO (1164) and DNA oxidative base modifications (1165), proteins offer some advantages as markers of oxidative stress. Proteins have unique biological functions, so there are unique functional consequences resulting from their modification (e.g., loss of clotting from oxidation of fibrinogen (1145), impaired ATP synthesis by oxidation of GAPDH (1166)). Products of oxidative protein modification are relatively stable and there are sensitive assays available for their detection; thus, from a purely technical perspective, they serve as suitable markers for oxidative stress. Importantly, the nature of the protein modification can give significant information as to the type of oxidant involved in the oxidation process. For example, chlorotyrosyl moieties and amino acyl adducts on lysine residues are probably specific markers of oxidation by HOCl, and hence reflect neutrophil and/or monocyte involvement in the oxidative stress (1167, 1168). Similarly, the presence of nitrotyrosyl residues in proteins indicates that nitric oxide and

superoxide (and hence peroxynitrite) were generated at the site of the damaged protein (1169). Carbonyls can be induced by almost all types of ROS and hence do not shed significant light on the source of the oxidative stress. In addition, carbonyls are relatively difficult to induce compared to methionine sulfoxide and cysteinyl derivatives and thus may be reflective of more severe cases of oxidative stress. Indeed, detection of elevated levels of protein carbonyls is generally a sign not only of oxidative stress but also of a disease-associated dysfunction (690, 1170). The highly specific nature of protein oxidation also leads to one of the disadvantages of using these macromolecules as markers of oxidative stress; that is, there is no single universal marker for protein oxidation. Because so many different protein oxidation products can be formed, it may be necessary to set up several different assays in order to find the most appropriate assay for the type of oxidative stress involved.

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