CHAPTER 1

Enzymes Involved in Redox Reactions: Natural Sources and Mechanistic Overview

1.1 MOTIVATION: GREEN CHEMISTRY AND BIOCATALYSIS

Current environmental concerns are pressuring Chemical and Pharmaceutical industries to develop novel synthetic approaches that may operate under more benign conditions. This trend has paramounted the appearance of the "Green Chemistry" as a core discipline, with an increasing importance both in academia and industry. In a nutshell, Green Chemistry—as well as Green Engineering—has been compiled under several principles, as stated by Anastas and Zimmerman, and Tang and coworkers [1,2]. From the Green Chemistry approach, these principles are gathered in the acronym "PRODUCTIVELY":

- Prevent wastes
- Renewable materials
- Omit derivatization steps
- Degradable chemical products
- Use safe synthetic methods
- Catalytic reagents
- Temperature, ambient pressure
- In-process monitoring
- Very few auxiliary substances
- E-factor, maximize feed in product
- Low toxicity of chemical products
- Yet, it's safe

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On the other hand, from the Green Engineering perspective, those principles are grouped in the acronym "IMPROVEMENTS":

- Inherently nonhazardous and safe
- Minimize material diversity
- Prevention instead of treatment
- Renewable material and energy inputs
- Output-led design
- Very simple
- Efficient use of mass, energy, space, and time
- Meet the need
- Easy to separate by design
- Networks for exchange of local mass and energy
- Test the life cycle of the design
- Sustainability throughout product life cycle

In this respect, the use of enzymes and whole-cells as biocatalysts for synthetic purposes (White Biotechnology) is an increasingly important field that may fit, in many cases, with all or some of these Green Chemistry principles. In fact, enzymatic living processes are often conducted under extremely mild reaction conditions, for example, neutral pH, or no need of high pressures or temperatures, which may provide energy savings for the overall process. Albeit biocatalytic processes are not always in line with all Green Chemistry principles (e.g., wastewater generation after downstream processing), they often provide advantages when compared to other chemical approaches. These assets have triggered the development of biocatalysis, reaching today the status of established technology, and occupying a prominent role as "synthetic organic chemistry tool" [3–16].

1.2 SOURCES OF BIOCATALYSTS

In the early stages of biocatalysis, plant tissues, and animal organs were the most important sources of enzymes, representing in the 1960s about 70% of the biocatalysts used for synthetic purposes [17,18]. The trend rapidly changed, and 20 years later most of industrially used enzymes were already being obtained from microbial sources. There are still some commercially available enzymes from animal origin, mostly hydrolases, accounting for approximately 10% of total of enzymes used at industrial level [19]. In this group, catalase from liver (EC 1.11.1.6), triacylglycerol lipase (EC 3.1.1.3), and trypsin from pancreas (EC 3.4.21.4) are the most relevant animal enzymes currently used, mainly in food industries [20,21]. Enzymes from vegetable origins, such as papain and cysteine proteases from papaya latex (*Carica papaya*, *Carica candamarcensis*), have industrial relevance as well, representing almost 5% of the market [17,22,23]. Other enzymes, such as invertase [24], peptidases [25], and

acid phosphatase [26] are produced *in vivo* in plant cell cultures, but their production involves highly complex and expensive processes, thus showing a limited use at industrial scale [27]. However, some glycoenzymes, such as glutamine synthetase, which are not easily produced as recombinant proteins in microbial hosts, are suitable candidates for the *in vitro* production with adequate cell lines [28].

While the origin of the biocatalysts can obviously be highly diverse, microorganisms are a rich source of enzymes, and thus their use as whole-cells, or the use of isolated microbial enzymes as biocatalysts has been vastly reported in the literature. Since the 1960s microbial enzymes have been replacing the biocatalysts from other origins, and to date, represent over 90% of the total market [17]. Currently, the exploitation of microbial diversity in the quest for new enzymes with novel activities is one of the major research goals in biocatalysis. This is complemented by the rational design of enzymes, and their production and overexpression in adequate microbial hosts through genetic engineering techniques [29]. Remarkably, the use of recombinant microorganisms was originally envisaged for the production of proteins of therapeutic interest. However, its real advantage is the reduction of production costs for a wide variety of proteins, especially when compared with the fermentation of wild-type microorganisms [30].

1.2.1 Plants and Animals as Sources of Redox Biocatalysts

As stated above, animal and plant tissues are classic sources of biocatalysts. Enzymes from higher eukaryotes have been traditionally used in food industry as food additives, in fruit processing or in wine production, as well as pharmaceutical additives. Some examples are the use of papain, lipoxygenase (LOX), or pepsin in processes already developed in the 1980s, which are still widely used [31,32]. Many hydrolases such as porcine pancreas lipase (PPL), pig liver esterase (PLE), or chymotrypsin and trypsin have been isolated from animal tissues, and have been widely used as biocatalysts [33]. Oxidoreductases (EC 1.-), hydrolases (EC 3.-), lyases (EC 4.-), and isomerases (EC 5.-) provide the vast majority of examples of higher eukaryotic enzymes for industrial applications, and no commercial processes using plant or animal enzymes from other enzyme classes have been reported [32].

Some of these animal or plant tissues can provide high amounts of enzymes (up to 1% of wet weight), for example, pancreatic enzymes or others involved in specific metabolisms (from liver or heart), or enzymes located in plant reserve organs such as seeds. However, recovering these enzymes from tissues is often cumbersome, and thus alternative sources must be found for their production at large scale for synthetic purposes. In addition, in case of pancreatic enzymes, after the discovery of pancreas as insulin-producing organ in 1921, the tissue became very expensive as a source of enzymes for biocatalysis. The enzymes that are still obtained from pancreas—trypsin and chymotrypsin—are actually by-products in insulin metabolism. Furthermore, nowadays insulin is mostly produced by recombinant microorganisms (*Escherichia coli* or yeast cells), as other enzymes originally obtained from pancreas do as well.

Higher eukaryotic enzymes usually need to be isolated and purified, or cloned and overexpressed in suitable hosts in order to obtain sufficient amounts for biocatalytic

applications. The use of synthetic host-adopted genes and codon-optimized *E. coli* strains, and the development of highly successful eukaryotic expression systems such as the yeasts *Pichia pastoris* and *Hansenula polymorpha*, have enabled the production of large quantities of eukaryotic enzymes within a short time [34].

Among redox enzymes from animal origin, horse liver alcohol dehydrogenase (HLADH, EC 1.1.1.1), or cytochrome P450 monooxygenases (CYP450, EC 1.14.-.-) are the most extensively used, both in academic and industrial settings [32]. CYP450s belong to a superfamily of hemeproteins with high catalytic versatility, which generally perform the monooxygenation of aliphatic compounds as a key step in the production of fine chemicals, and catalyze the metabolism of a wide variety of endogenous and exogenous compounds (see Section "Heme iron monooxygenases cytochromes P450"). They are involved in reactions as diverse as hydroxylations, *N*-, *O*-, and *S*-dealkylation, sulfoxidation, epoxidation, deamination, desulfuration, dehalogenation, peroxidation, and *N*-oxide reduction (see Chapter 4) [35–43]. Their substrates include fatty acids, steroids, prostaglandins, and a number of exogenous compounds such as drugs, anesthetics, pesticides, and carcinogens [35]. This diverse catalytic potential attracted researchers from different fields to study cytochrome P450 systems. Industrial applications of human P450s involve two different objectives: the production of active pharmaceutical intermediates and the simple and fast production (in mg-scale) of metabolites for drug development. Human CYP450 has been expressed in different systems such as mammalian cells, yeasts, and bacteria such as *E. coli* [32]. For this application, *E. coli* expression systems are the easiest and less expensive to operate, yielding high quantities of recombinant proteins. At present, complete sets of ready-to-use liver enzymes expressed in *E. coli* are commercially available, providing kits with all six major human liver cytochromes for simple application. With regard to HLADH, three groups of isoenzymes with different substrate specificities are known, each one containing one main form [44–47]. The enzyme is a dimer, and these three forms correspond to the possible dimeric combinations of two protein chains [48–50] that are not interconvertible, therefore having different primary structures. The three isoenzymes were crystallized [51,52], and are similar in their amino acid analysis [51], but distinguishable by immunological methods [53]. Regarding synthetic applications of HLADH, its ability for the stereoselective reduction of a broad range of carbonyl compounds is outstanding, including aromatic, open-chain, or cyclic ketones, and α- or β-ketoesters (see Chapter 3).

Apart from the above-described two main enzymes, bovine liver glutamate dehydrogenase (GluDH, EC 1.4.1.2) is another example of dehydrogenases from higher eukaryotes [32]. The enzyme reversibly catalyzes the reductive amination of α-ketoglutarate to L-glutamate using NADH as cofactor. The crystal structure of this homohexameric mitochondrial enzyme has been solved at 2.8 Å resolution and its catalytic mechanism has also been demonstrated [54].

Plant cells exhibit an ample potential for the biosynthesis of secondary metabolites [55]. Although in cell cultures the formation and accumulation of these metabolites do not occur, such cultures retain the ability to transform exogenous substrates into products of interest. Thus, plant cells have been widely used in biocatalysis, as isolated enzymes as well as whole-cells (cell cultures or crude parts such as leaves, roots, seeds,

etc.), either free or immobilized, displaying hydrolase and oxidoreductase activities [55]. When performing biotransformations mediated by "native" plant organs or tissues, the main drawback is the lack of reproducibility of the experiments. Some biochemical features of these biocatalysts can vary easily depending on several factors, such as the origin of the plant, the season of the year, or particular climatic conditions [56]. Likewise, other drawbacks are the localization and concentration of enzyme in the cell, and the presence of side reactions leading to undesired by-products, or enzymes that could degrade the desired products. In addition, another issue is the mass transfer limitations involved in the transport between the bulk medium and the enzyme. Overall, these problems contribute to the often observed low efficiency of these processes [55]. Strategies such as elicitation, permeation of the cells by the addition of dimethyl sulfoxide (DMSO) or organic alcohols to promote the substrate uptake and product release, or the variation of the pH, have been developed to improve efficiencies. Cyclodextrins were also used as additives in cases of water-insoluble or poorly soluble substrates because they are able to form inclusion complexes with a variety of apolar ligands [57]. Another major drawback of cultured cells as biocatalysts is the somaclonal variation, which may lead to unstable biochemical behavior [58]. This problem can be circumvented either by the continuous screening to maintain productive lines, or by the use of organized tissues, such as roots or root cultures, which are able to provide biotransformations with potential applicability for the production of added-value products, and even for scaling up purposes. In this context hairy roots, which are obtained by the integration of a region (T-DNA) of the Ri plasmid of the bacterial soil pathogen *Agrobacterium rhizogenes* to the plant genome [59], have been used as biocatalysts.

The use of dehydrogenases from vegetal origin has been widely exploited for the reduction of carbonyl groups to the corresponding alcohols. A broad range of structurally diverse carbonyl compounds including aromatic and aliphatic ketones, diketones, ketoesters, aldehydes, steroids, alkaloids, terpenoids, coumarins, and lignans, among others, can undergo redox reactions catalyzed by plant dehydrogenases (see Chapter 3) [55,56,60–77]. Among crude plant cells or plant cell cultures used as sources of dehydrogenases, *Daucus carota* has been one of the most widely used [60,62–71,75,76,78]. Cells of *Raphanus sativus* [56], *Passiflora edulis* [77], *Cocos nucifera* [61], celeriac (*Apium graveolens*) [62,63,70], horseradish (*Armoracia lapathifolia*) [62], legumes such as *Pisum Sativum* [73], *Phaseolus angularis* [72], and *Phaseolus aureus* [74], among others [79–82], have been also used as biocatalysts in dehydrogenase-catalyzed reductions or oxidations. Likewise, *R. sativus* hairy roots were used in the stereoselective reduction of a series of prochiral alkylaryl ketones. Most of the reactions proceeded with high yields and excellent enantioselectivities [56]. Hairy roots of *Brassica napus* [83] and *D. carota* [84] were also employed as biocatalysts for the stereoselective reduction of aromatic and aliphatic ketones, diketones, and ketoesters.

In addition to the vast group of dehydrogenases, other enzyme types of vegetal origin are of synthetic use, such as oxidases and peroxidases. Glycolate oxidase (EC 1.1.3.15) is a peroxisomal oxidase isolated either from mammals including pig liver, rats and humans, or from green plants such as spinach leaves, pea, cucumber,

and pumpkin [32]. Of all of them, spinach redox enzymes displayed the highest yields also having the best specific activity for the oxidation of glycolic acid to glyoxylic acid [85]. The biocatalytic oxidation was performed in high yields (>99%) and resulted in a few undesirable by-products, in the presence of oxygen and ethylenediamine, using both glycolate oxidase and catalase. Importantly for practical purposes, spinach glycolate oxidase is also active for the oxidation of other α-hydroxy carboxylic acids [86]. Yet, purified enzymes from spinach leaves are not suitable for stable enzyme preparations for industrial applications [32]. Therefore, its gene was cloned and overexpressed in *E. coli* [87,88], *Saccharomyces cerevisiae* [89], and *P. pastoris* [90]. This was the first example of expression of a plant gene in *P. pastoris*, and also the first engineered *P. pastoris* as a whole-cell catalyst developed for a commercial bioprocess. The protein crystal structure was determined at 2.0 Å resolution, showing a catalytically active tetramer or octamer made up of identical 40 kDa subunits, which form an eight-stranded α/β barrel [91]. Optically pure (*R*)-2-hydroxyacids were also obtained on semipreparative scale with >99% *ee* and good-to-excellent conversions by α-hydroxylation of long-chain carboxylic acids with molecular oxygen, through a reaction catalyzed by an α-oxidase from peas (*P. sativum*) [92].

Finally, the importance of peroxidases is emphasized by their wide distribution among living organisms and by their multiple physiological roles. They have been divided into two main superfamilies according to their source and mode of action: plant (nonanimal) peroxidases and animal peroxidases (see Section 1.3.4) [93]. The plant peroxidases superfamily, which contains enzymes from both prokaryotic and eukaryotic origin, can be in turn divided in three classes, based on structural similarities and in a suspected common evolutionary origin [94]: peroxidases from prokaryotic origin (Class I), fungal peroxidases (Class II), and plant peroxidases (Class III). Horseradish peroxidase (HRP, EC 1.11.1.7), peanut peroxidase (PNP) [95], soybean peroxidase (SoP) [96,97], tobacco peroxidase (TobP) [98], tomato peroxidase (TomP) [99], and barley peroxidase (BaP) [100] are examples of Class III peroxidases. They contain an *N*-terminal signal peptide for secretion, two conserved calcium ions, four conserved disulfide bridges, an extra helical region that plays a role in access to the heme edge, and a carbohydrate content between 0% and 25% [93]. Of all of them, HRP is the most intensively studied peroxidase from plant origin, catalyzing a variety of reactions such as reduction of hydroperoxides, epoxidation, sulfoxidation, halogenation, and oxidation of phenols and aromatic amines (see Chapter 5).

A general strategy to improve the biocatalytic efficiency is the biocatalyst immobilization [101]. Whole-cells as well as isolated enzymes can be immobilized in order to overcome stability problems and to enable the biocatalyst reuse for cost reduction. Immobilized whole-cells have additional advantages over freely suspended cells. General methods for immobilization of plant cells are gel entrapment by ion exchange, precipitation, polymerization, and by fixing them into preformed structures [102]. Enzymes can be adsorbed into insoluble supports by hydrogen bonding, dipole–dipole interactions, or hydrophobic interactions. Most commonly used supports are polypropene (e.g., Accurel TM), and diatomaceous earth (Celite). Immobilization of enzymes by ion exchange is possible when the optimum pH of the enzyme is not

close to its isoelectric point. Polyacrylamides are common matrixes used for covalent linking of the enzymes. High degrees of cross-linking prevent leakage and loss of the biocatalyst, but are not suitable for bulky substrates. Microencapsulation, forming a microsphere of polymeric membranes around the enzyme in solution, is another frequently used method [55].

1.2.2 Wild-Type Microorganisms

Microorganisms have been widely exploited as biocatalysts in the area of medicine, agriculture, and food industry, and their industrial applications have an increasing impact. Actually, as mentioned before, most of the enzymes currently used in industry have microbial origin. Since the beginning of biotechnology, microbial wild-type strains have been used for food and beverages production. In turn, native isolated enzymes from microbial origin, as well as recombinant proteins and microorganisms, are having an increasingly widespread use in pharmaceutical, chemical, or biofuels industries, being mainly designed through genetic engineering [103].

1.2.2.1 Yeasts The large-scale use of yeasts in enzyme-catalyzed processes dates back several centuries ago, with the production of ethanol from glucose in alcoholic beverage manufactures. *S. cerevisiae* (baker's yeast, BY) was often the microorganism of choice, mainly due to its wide availability and low cost. Moreover, BY does not need sterile growth media, and remains viable and easy to work in a nonmicrobiology-specialized laboratory. In addition to these desirable features, as biocatalyst, baker's yeasts (as well as other yeasts) can be used in chirality generation, in racemic resolutions, or in the regioselective conversion of functional groups. For such purposes, besides redox-related enzymes (mainly dehydrogenases), other biocatalytic yeast enzymatic systems include hydrolytic enzymes (lipases, epoxide hydrolases) and lyases for C–C bond formation. Clearly, *S. cerevisiae* is still to date the most widely used yeast biocatalyst [104].

Among redox enzymes, dehydrogenases are versatile and powerful biocatalysts for synthetic organic chemistry, mainly involved in the reduction of carbonyl groups—generating chiral alcohols—or in the asymmetric reduction of enones or imines. On the other hand, yeast dehydrogenase-mediated oxidations generally involve the destruction of chiral centers, and thus their practical use has been mainly guided by environmental considerations, a major advantage over conventional chemical oxidations (see Chapter 3). In the reduction of carbonyl groups—usually associated to the synthesis of (*S*)-secondary alcohols—most of the yeast dehydrogenases follow the so-called Prelog's rule [105]. For these purposes, yeast dehydrogenases can be used either as whole-cells (wild type or recombinant), or isolated enzymes with the adequate cofactor supply and a suitable recycling method [106]. As mentioned above, *S. cerevisiae* is the most widely used yeast as biocatalyst, being efficient for selectively reducing monocarbonylic compounds (aldehydes and ketones) with alkyl or aryl substituents and dicarbonylic compounds (cyclic and acyclic α- and β-diketones, α- and β-ketoesters), thus obtaining (*S*)-secondary alcohols (see Chapters 3 and 7). Sterically hindered ketones are not usually substrates for

yeast dehydrogenases except with the exception of methyl ketones (see Chapter 3). Apart from baker's yeast, a wide number of oxidoreductases from *Candida* sp. have also been characterized and used as biocatalysts [107]. These enzymes are rather diverse, enabling not only highly enantioselective reductions of carbonyl groups or deracemizations, but also some promiscuous catalytic imine reductions. In addition, some *Candida* sp. dehydrogenases have been extensively used for cofactor regeneration purposes, with the aid of sacrificial substrates.

1.2.2.2 Fungi Fungi have traditionally been one of the most studied whole-cell enzymatic systems as biocatalysts [108]. They can be identified in nature through the screening of a wide variety of environments and habitats. Bioprospecting for new biocatalysts even in extreme environments—thermal, cold, or hypersaline ecosystems—can lead to the discovery of new fungal enzymes able to catalyze a wide variety of synthetically interesting reactions. Among the most extensively explored fungal enzyme systems for biocatalytic purposes, redox enzymes can be highlighted, catalyzing oxygenase- as well as peroxidase-mediated hydroxylations, sulfoxidations, epoxidations and Baeyer–Villiger oxidations, dehydrogenase-catalyzed stereoselective reductions, oxidations or deracemizations [104,108].

Fungal dehydrogenase-catalyzed reactions, in particular of carbonyl compounds to furnish alcohols regio- and enantioselectively, are the most widely described in the literature. The broad substrate specificity of these enzymes allows for the preparation of structurally different compounds, including aromatic, cyclic, open chain aliphatic (*R*)- or (*S*)-secondary alcohols, or α- or β-hydroxyesters and hydroxyketones [108]. Dehydrogenase-catalyzed racemization or deracemizations are also efficient strategies for interconversion of enantiomers, with a number of fungal enzymes reported for these types of reactions [108].

Oxygenase-, oxidase-, and peroxidase-mediated oxidations, introducing oxygen atoms into nonactivated hydrocarbon chains of organic compounds, are useful for organic synthesis as well. These fungal enzymes catalyze Baeyer–Villiger reactions [109], epoxidations [110], sulfoxidations [111], and hydroxylations [112] using growing or resting whole-cells, as well as isolated enzymes. Likewise, fungal laccases catalyze oxidation and coupling reactions [113,114]. Although these enzymes are widely distributed in fungi, higher plants, bacteria and insects, the majority of laccases characterized so far are of fungal origin, spread in more than sixty strains belonging to various classes such as *Ascomycetes*, *Basidiomycetes*, and *Deuteromycetes* [115]. Especially, white rot *Basidiomycetes* are efficient lignin degraders, being a valuable source of laccases. Moreover, fungi are also an important source of peroxidases. Due to the catalytic versatility and stability of these enzymes, fungal peroxidases are, together with fungal laccases [114,116], of particular interest as potential biocatalysts for redox processes at industrial level [93,117–119].

1.2.2.3 Bacteria The variety of bacterial genera and species, and the different enzyme types according to their metabolism, role and environment, make these microorganisms a rich source of biocatalysts. Bacteria used in biocatalysis belong to a

wide variety of genera, including *Escherichia*, *Rhodococcus*, *Bacillus*, *Lactobacillus*, *Nocardia*, *Pseudomonas*, *Acinetobacter*, *Alcaligenes*, *Corynebacterium*, and so on [30,33]. Similar to enzymes from eukaryotic cells, bacterial enzymes can be used both as isolated and as whole-cells, and either free or immobilized. Due to management requirements in the laboratory, the use of isolated enzymes is mainly restricted to extracellular and cofactor-independent-enzymes, such as hydrolases, whereas the whole-cells of microorganisms are preferred in synthetic procedures involving cofactor-dependent or intracellular enzymes. However, at industrial level examples of redox enzymes comprise both isolated enzymes and whole-cells (see Chapters 7 and 8).

Bacterial enzymes or whole-cell-catalyzed biotransformations are used in the industrial preparation of a variety of compounds [33]. The use of redox enzymes from bacterial origin for the preparation of chiral intermediates for fine chemicals is highly widespread. Dehydrogenases, involved in the reduction of carbonyl groups or the enantioselective oxidation of alcohols, as well as mono- and dioxygenases for hydroxylations of arenes and unfunctionalized alkanes, Baeyer–Villiger reactions and alkene monooxygenation, are outstanding examples [104,120]. Most of the monooxygenases used in biocatalysis are from bacterial origin, and aromatic dioxygenases have only been found in bacteria, mainly in genus *Pseudomonas*, *Sphingomonas*, and *Rhodococcus*, being intracellular enzymes. Although to date many oxygenases are known and thoroughly described, their use for synthetic purposes is still limited due to their low availability in large quantities, instability, and high cost of the required cofactors. Moreover, many of them are membrane-associated proteins, reducing their synthetic potential and hampering their use as isolated enzymes. However, efforts have been done to overcome these drawbacks, for example, using recombinant whole-cells overexpressing the desired enzymes (see Chapter 4).

1.2.3 Metagenomic Assessments

An ample range of microbial diversity, not yet accessed or explored, might be a valuable source for possible novel biotechnological applications. In this respect, only 0.1–10% of natural biodiversity can be cultured under conventional laboratory conditions [121,122]. Metagenomics, that is, the genomic reconstruction of uncultivable microorganisms that emerged in the late 1990s, refers to the extraction of the entire genetic material from all organisms present in an environmental sample (metagenome) [123,124]. It involves the culture-independent analysis of the collective microbial genomes contained in the metagenome by using two approaches: (i) the function-driven analysis and (ii) the sequence-driven analysis, to extract biological information from metagenomic libraries [125–127]. The function-driven analysis identifies the clones that express a desired feature, and then characterizes the active ones by sequencing and biochemical analysis, rapidly finding clones with potential applications. The limitation of the method is that it requires the expression of the function of interest in the host cell and the clustering of all of the genes required for the function. It also depends on the availability of an adequate assay for the function of interest that can be performed on vast libraries, because the frequency

of active clones is quite low. To overcome these limitations, improved systems for heterologous gene expression have been developed with shuttle vectors that facilitate screening of the metagenomic DNA in diverse host species and with modifications of *E. coli* cells to expand the range of gene expression. Conversely, sequence-driven analysis is based on the use of conserved DNA sequences to design hybridization probes or polymerase chain reaction (PCR) primers to screen metagenomic libraries for clones that contain sequences of interest. Significant discoveries have resulted from random sequencing of metagenomic clones [127]. Metagenomics relies on the efficiency of four main steps, which are: (i) the isolation of genetic material, (ii) its manipulation, (iii) the transfer of the genetic material into a surrogate organism to create a metagenome clone library, and (iv) the analysis of the genetic material in the metagenomic library [121,122,128]. The information about diversity and community structure of microbes is further obtained through the corresponding metagenome library sequency [129,130]. The specific activities within the metagenome can be screened for particular enzymes, either *via* DNA sequences or enzymatic functions [131,132].

Metagenomic libraries have been screened for enzymes [133], and a number of metagenomic biocatalysts displayed novel substrate ranges and high stability under extreme conditions, improving their potential for industrial applications [134]. Many oxidoreductases have been incorporated into the redox-biocatalytic toolbox through metagenomic assessment [134]. As some examples, in the quest of microorganisms accepting 4-hydroxybutyrate, five clones were found displaying novel 4-hydroxybutyrate dehydrogenase activity [135]. The genes involved in metabolism of poly-4-hydroxybutyrate were also successfully screened in environmental libraries [136]. Short-chain dehydrogenases/reductases were found with <35% similarity to known enzymes, and thus could not have been detected using hybridization-based techniques such as PCR. Likewise, alcohol dehydrogenases (ADHs) oxidizing short chain polyols were obtained from twenty-four positive clones and characterized [137,138]. Polyphenol oxidase (PPO; tyrosinase), catechol oxidase, as well as multicopper oxidases, oxygenases, laccases, and L-amino acid oxidases (LAAOs) were also isolated from marine metagenome [139,140]. Clones capable of indirubin and indigo production in *E. coli* were isolated from forest soil metagenome [141]. A novel ADH, $ADHE_{Meta}$, was isolated from a waste-water treatment metagenomic library [142]. Although this enzyme showed a reasonably high sequence identity to the well-characterized ADHs from *E. coli* (60%) and *Clostridium acetobutylicum* (61%)—which catalyze the ethanol and butanol formation in a two-step reductive process—it was not deactivated by oxygen [123]. This functional property suggests that the *in vivo* role of the enzyme was catabolic, rather than the typical anabolic role of ADHs.

1.3 OVERVIEW OF REDOX ENZYMES

Enzymes catalyzing oxidation–reduction reactions—oxidoreductases, EC 1.—always act on their substrates involving an electron transfer. These biocatalytic

redox processes have been attracting an increasing interest for synthetic organic chemistry, especially for applications in the chemical and pharmaceutical industries [143]. Besides the inherent enantioselectivity of enzymes, other challenging chemical transformations like the regioselective introduction of oxygen from O_2 into chemically inert C–H bonds can be performed. Furthermore, oxidoreductases catalyze the formation of chiral products from prochiral substrates with a theoretical 100% yield, as well as other reactions such as the oxifunctionalization of C–C double bonds and C–N bonds, Baeyer–Villiger oxidations, and the oxidation of alcohols, aldehydes, acids, and aromatic compounds, or else the reduction of ketones, aldehydes, C–C and C–N double bonds, and reductive aminations [120,144,145]. Applications of these enzymes comprise mainly asymmetric oxyfunctionalization of nonactivated hydrocarbons and enantioselective reductions of carbonyl compounds, synthesis and modification of polymers, as well as oxidative degradation of pollutants and construction of biosensors for a variety of analytical and clinical applications [146].

Presently, more than 25% of the known enzymes are oxidoreductases [147,148]. Due to their biodiversity and different activities, oxidoreductases have been classified according to the nature of the oxidizing substrate as dehydrogenases, oxygenases (monooxygenases and dioxygenases), oxidases and peroxidases (Figure 1.1). Alternatively, they can be classified according to their coenzyme requirements [149]. Peroxidases are sometimes considered as a subset in the group of oxidases [106], but taking into account the differences in terms of their catalytic mechanism, as well as their synthetic applications, in this book peroxidases will be treated as independent enzymes.

Dehydrogenases catalyze reversible redox reactions and are thus used as oxidative or reductive biocatalysts. Conversely, oxygenases, oxidases and peroxidases catalyze irreversible oxidations due to the reaction thermodynamics. This is attributed to the highly exothermic reduction of O_2 or H_2O_2, which act as electron acceptors in the case of oxygenases and oxidases, or peroxidases respectively [144]. In contrast to oxidases, oxygenases incorporate one (monooxygenases) or both (dioxygenases) oxygen atoms of O_2 into their substrates.

Redox enzymes often require cofactors, which are direct products of the primary metabolism of the cells. These requirements impose special constraints on the development of bioprocesses involving oxidoreductases. These cofactors or coenzymes, such as NAD(P)H or $NAD(P)^+$, act as electron donating or accepting molecules, being coupled to the redox metabolism within cells [14,150–153]. Therefore an efficient cofactor recycling system is required to achieve economically efficient processes (see Chapters 2 and 7–8 for applying biocatalysis at practical level) [104,154–158]. Exceptions include peroxidases, which couple the reduction of hydrogen peroxide to water with the two electron oxidation of the substrate, and oxidases, which couple the reduction of O_2 to hydrogen peroxide or water with the two- or four-electron oxidation of an activated carbon scaffold. All reactions dealing with O_2 or H_2O_2 reduction involve reactive oxygen species such as oxygen radicals or hydrogen peroxide itself, which often cause extensive damage to biomolecules, including enzymes. These reactive oxygen species and their damage can be handled by the metabolism

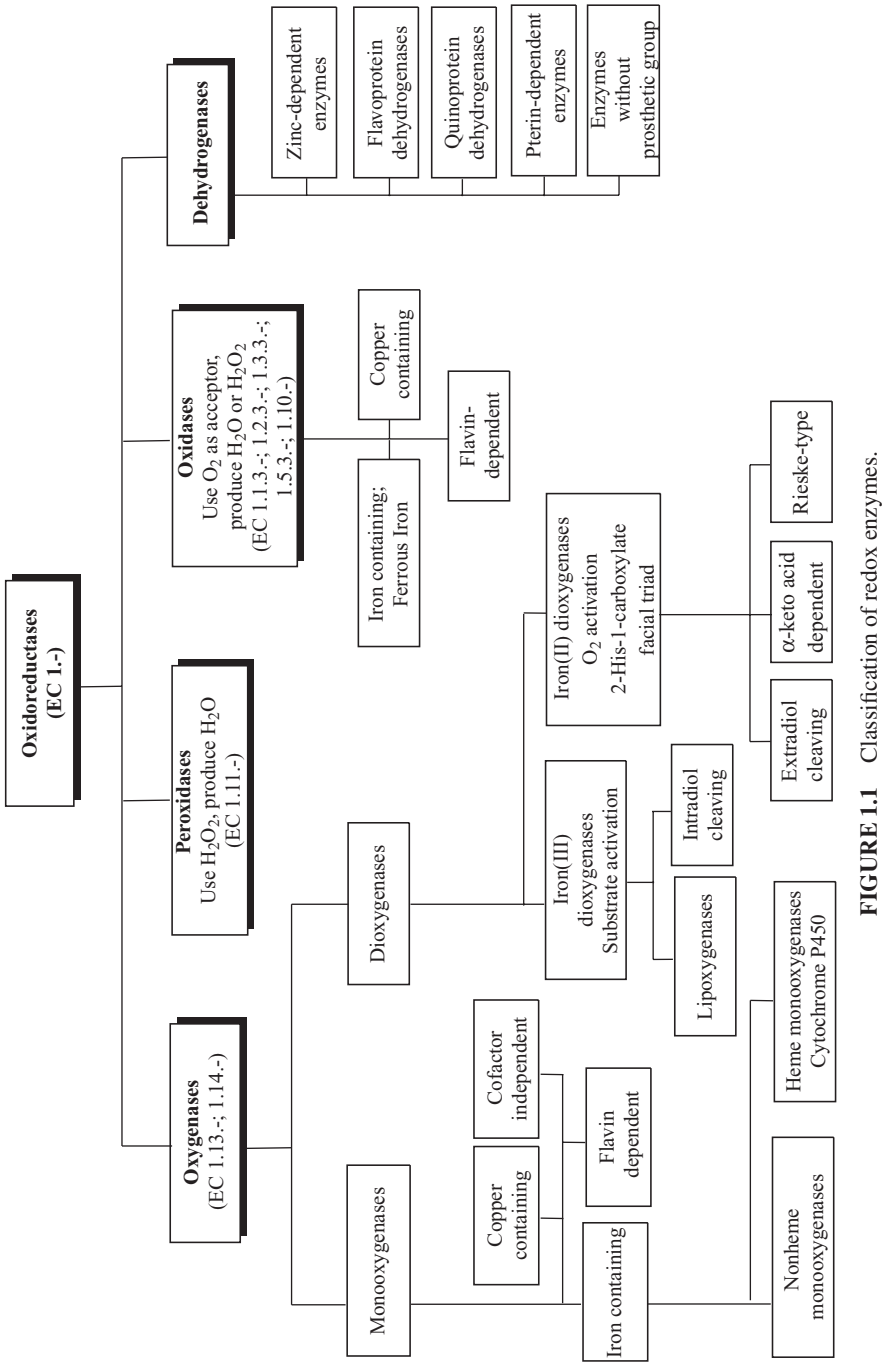

FIGURE 1.1 Classification of redox enzymes.

of resting or growing cells, an advantage that can be exploited for productive redox biocatalysis [144].

1.3.1 Dehydrogenases

Dehydrogenases (also known as reductases) are the most widely used oxidoreductases for synthetic purposes [104,143,158–160], and they are classified in groups EC 1.1.1.- (acting on the CH–OH group of donors), EC 1.2.1.- (acting on the aldehyde or oxo group of donors), or EC 1.4.1.- (acting on the CH–NH_2 group of donors). They always use NAD^+ or $NADP^+$ as cofactors [159].

Regarding substrate specificity of dehydrogenases, enantiopure alcohols, hydroxyacids, hydroxyesters, amines, and amino acids can be obtained through dehydrogenase-catalyzed reductions of the corresponding prochiral compounds [104,158,159,161]. The enzymes catalyze the reversible reaction, in which one atom of hydrogen and two electrons of the donor species are transferred to the C-4 position in the nicotinamide of the cofactor (NAD^+ or $NADP^+$). The reverse reaction involves the transfer of one atom of hydrogen and two electrons from the reduced form of the nicotinamide cofactor (NADH or NADPH) to the carbonyl (or imino) group, resulting in the reduction of the substrate, being often a highly specific reaction. The hydrogen atom transfer occurs either on the *Si* face or on the *Re* face of the carbonyl group, resulting in the corresponding (*R*)- or (*S*)-alcohol, respectively. Both, NADH and NADPH have two diastereotopic hydrogen atoms (*pro-R* and *pro-S*), which can be transferred as a hydride to an oxidized substrate (aldehyde, ketone, or imine). In turn, these substrates have two diastereotopic or enantiotopic faces (*Re*-face or *Si*-face) in the sp^2 carbon atom that will be reduced. Theoretically, any NAD(P)H-dependent redox reaction can occur stereospecifically *via* any of the four possible pathways to generate any isomer on the substrate (Figure 1.2).

In practice, most ADHs catalyze the transfer of the *pro-R* hydrogen atom at C-4 of the nicotinamide cofactor to the *Re* face of the carbonyl substrate, furnishing the corresponding (*S*)-alcohol as the main reduction product. This general behavior

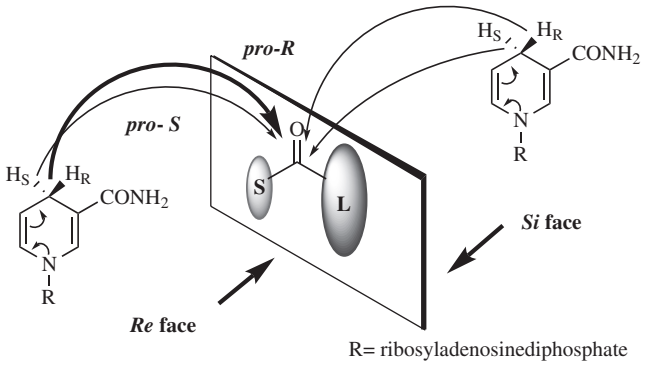

FIGURE 1.2 Stereochemical possibilities for the hydride transfer in the dehydrogenase-mediated reduction of carbonyl compounds. **L**, large substituent; **S**, small substituent.

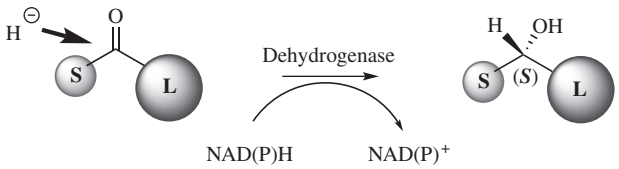

FIGURE 1.3 Stereochemical preference for the hydride transfer in the dehydrogenase-catalyzed reduction of carbonylic compounds, according to Prelog's rule. **L**, large substituent; **S**, small substituent.

is described by the Prelog's rule [105], where the stereochemical course of the reaction is predictable, and largely depends on the steric requirements of the substrate (Figure 1.3). Depending on the binding site of the cofactor in the enzyme, either the *pro-R* or *pro-S* hydrogen at C-4 of the nicotinamide is abstracted. The Prelog's rule is an empirical rule based on the stereochemistry of microbial reductions using whole-cells of *Curvularia falcata*.

Dehydrogenases can be classified into five categories depending on their prosthetic group: (i) zinc-dependent enzymes, (ii) flavoprotein dehydrogenases, (iii) pterin-dependent enzymes, (iv) quinoprotein dehydrogenases, and (v) dehydrogenases without prosthetic groups [144].

1.3.1.1 Zn-Dependent Dehydrogenases Zinc-dependent dehydrogenases comprise mainly ADHs that are grouped according to their substrate spectrum and structure into long-, medium- and short-chain ADHs [162]. These enzymes catalyze the (reversible) oxidation of primary and secondary alcohols to the corresponding aldehydes and ketones respectively, often in a chemo-, regio-, and stereoselective way. For synthetic purposes, the most relevant enzymes of this group are HLADH and ADH from *Thermoanaerobacter brockii* (TbADH).

Structure–function studies have been undertaken for the Zn-dependent medium-chain HLADH, revealing a nucleotide binding domain and a catalytic domain, with the catalytically active zinc buried between them [163]. In HLADH, the zinc is tetra-coordinated to three conserved residues (Cys46, His67 and Cys174) of the active site, and to a water molecule [164]. There are two main mechanisms proposed for HLADH-catalyzed alcohol oxidation, which differ in the coordination of the zinc atom during the redox process. One proposes that the zinc ion stays in tetrahedrical coordination during catalysis, as the bound water molecule is replaced by the hydroxyl group of the alcohol. Then, two hydrogen atoms are removed from the substrate by a coupled process of proton abstraction and hydride ion transfer. The intermediate turns to the corresponding oxidation product, with the concomitant hydride transfer to $NAD(P)^+$ to complete the reaction. The other mechanistic proposal suggests a change in the coordination symmetry of the zinc, leading to a pentacoordinated species, by the binding of the substrate in addition to the water molecule, and followed by the same steps than in the previously proposed mechanism. Recent X-ray studies on HLADH and TbADH provided evidence for the latter mechanism, involving a pentacoordinated zinc during catalysis [165,166]. Kleifeld and coworkers proposed

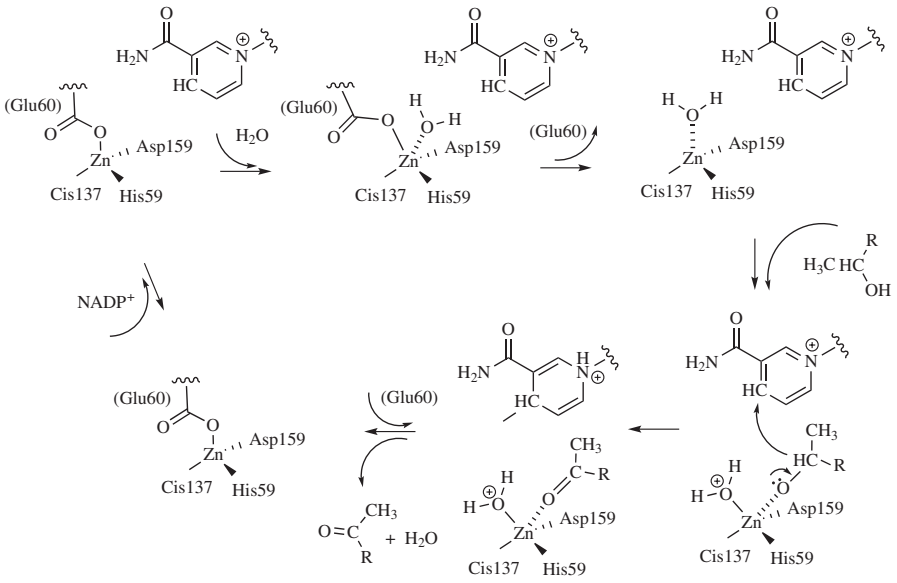

FIGURE 1.4 Proposed mechanism for the oxidation of secondary alcohols mediated by alcohol dehydrogenase from *Thermoanaerobacter brockii* (TbADH).

that TbADH differs from other ADHs, since the catalytic Zinc is bound to three conserved residues (Cys37, His59, and Asp159), and a residue of glutamic acid (Glu60) instead of water in the forth coordination site. This Glu60 is exchanged by a water molecule during the catalysis, generating a pentacoordinated intermediate (Figure 1.4).

1.3.1.2 Flavin-Dependent Dehydrogenases Enzymes containing flavin adenine dinucleotide (FAD) as prosthetic group, either covalently or noncovalently bound, known as flavoprotein dehydrogenases, are mostly involved in O_2 activation reactions. They are important enzymes in redox metabolism, yet less used for synthetic applications [144]. As in the case of other flavin-dependent enzymes such as oxidases, FAD serves as an electron acceptor during the oxidation of the substrate. Within this group, acyl-CoA dehydrogenases are a family of flavin-dependent enzymes whose members are highly conserved in sequence, structure, and function. These enzymes catalyze key steps in the mitochondrial oxidation of lysine, hydroxylysine, and tryptophan. Glutaryl-CoA dehydrogenase (GCD, EC 1.3.99.7) has a homotetrameric structure containing one noncovalently bound FAD in each monomer, and catalyzes the oxidative decarboxylation of glutaryl-CoA to crotonyl-CoA [167]. The reductive half reaction generates the enzyme-bound intermediate glutaconyl-CoA and CO_2 (Figure 1.5). Upon binding the substrate glutaryl-CoA, a glutamate residue functioning as catalytic base abstracts an α-proton from the substrate, followed by hydride transfer from the C-3 position of the substrate to the flavin, yielding the two electron reduced form of FAD and the enzyme-bound desaturated

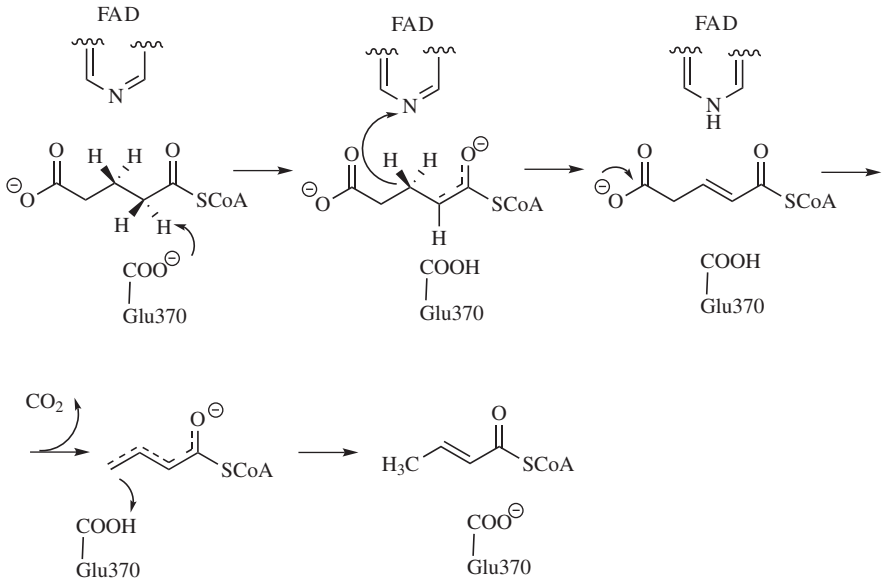

FIGURE 1.5 Proposed mechanism for the oxidative decarboxylation of glutaryl-CoA to crotonyl-CoA (human GCD numbering).

intermediate glutaconyl-CoA [168,169]. Subsequently, the latter is decarboxylated resulting in the formation of a dienolate anion and CO_2. The dienolate anion is protonated *via* proton transfer to C-4 from the catalytic base Glu370, which exchanges its proton with solvent water, giving rise to the crotonyl-CoA product (numbering of the residues is for the human GCD) [170]. The two electron reduced form of FAD is reoxidized in two consecutive steps, transferring the electrons to the respiratory chain.

Besides flavoproteins containing only the flavin in the catalytic center, there are also two-component dehydrogenases, having a covalently bound flavin in one subunit and a cytochrome in the other [171]. Examples for hydroxylating flavocytochromes include *p*-cresol dehydrogenase [172,173], and eugenol dehydrogenase [174], found in *Pseudomonas fluorescens* strains and denitrifying bacteria. After a fast electron transfer from the flavin to the cytochrome, *p*-cresol dehydrogenase transfers the electron to azurin or nitrate as physiological electron acceptors. During the reaction, a proton is removed from the hydroxyl group of the substrate *p*-cresol and a hydride ion is transferred from the methyl group to the N-5 atom of the covalently bound flavin. The formed quinone methide intermediate undergoes a nucleophilic attack of water at the methylene group, yielding *p*-hydroxybenzyl alcohol.

1.3.1.3 Pterin-Dependent Dehydrogenases Pterin-dependent dehydrogenases also belong to the group of hydroxylating enzymes, which use water as electron donor. They contain a so-called molybdopterin cofactor, where molybdenum is usually coordinated to the two thiol groups of a pyranopterin-based cofactor [144].

These enzymes can be composed of a variable number of subunits and contain different additional cofactors such as hemes, iron–sulfur clusters, and flavins, which transfer electrons from the molybdopterin cofactor to an electron acceptor. These molybdoenzymes catalyze the hydroxylation of activated carbon atoms, such as heteroaromatic ring carbons. Furthermore, selenium has been reported to participate in reactions catalyzed by xanthine dehydrogenase [175] and nicotinic acid dehydrogenases of anaerobic strains [176], for which NAD^+ and $NADP^+$ serve as electron acceptors, while nicotinic acid dehydrogenases from aerobic strains are selenium independent [177]. Molybdenum-enzymes are found in nearly all organisms, with *Saccharomyces* as a prominent eukaryotic exception [178]. The reaction mechanism has been characterized for xanthine dehydrogenase. A molybdenum center (as Mo(VI) in the resting state) with a water-derived oxo- or hydroxyl group in the ligand sphere is reduced to Mo(IV) during the substrate oxidation. Electrons derived from substrate hydroxylation are transferred through a [2Fe–2S] cluster and FAD to NAD^+ [179].

1.3.1.4 Quinoprotein Dehydrogenases Quinoproteins contain an amino acid-derived *o*-quinone as a cofactor. Different kind of quinone cofactors are known, but only pyrroloquinoline quinone (PQQ), which is not covalently bound to the enzyme, is involved in oxidoreduction reactions [180]. Quinoprotein dehydrogenases are highly interesting enzymes regarding redox metabolism, transferring reducing equivalents directly to the bacterial aerobic respiratory chain [144]. These enzymes are located in the periplasm, and are mainly involved in the dehydrogenation of primary or secondary hydroxyl groups in alcohols and sugars. These enzymes are frequently applied in biosensors [181]. Mainly two dehydrogenases of this class, in which PPQ is the only cofactor, have been extensively studied [182,183]. These are the membrane-bound quinoprotein glucose dehydrogenase (m-GDH, EC 1.1.5.2) and the soluble methanol dehydrogenase (s-MDH, EC 1.1.1.244), which catalyzes the oxidation of methanol to formaldehyde. Glucose dehydrogenase was found in a variety of bacteria including Gram-negative facultative anaerobes such as enteric bacteria and *Zymomonas*, as well as aerobic bacteria such as *Pseudomonas* and acetic acid bacteria [184]. The enzyme was originally investigated in *Acinetobacter calcoaceticus* in early 1960s by Hauge [185–187], and subsequently in late 1970s by Duine et al. [188], who reported that the enzyme was a quinoprotein. Mechanistic studies on s-MDH showed that upon substrate binding, the oxidation is initiated by an aspartate residue located in the active site, which is responsible for the abstraction of the proton from the alcohol group [189]. Then, a Ca^{2+} ion coordinated to the C-5 of the cofactor acts as a Lewis acid during substrate oxidation, stabilizing the C-5 carbonyl atom for the electrophilic attack by the hydride [144]. The proton abstraction is followed by a direct hydride transfer from the methyl group of methanol to the C-5 carbonyl of PQQ, followed by tautomerization to $PQQH_2$ [190]. The oxidized product, formaldehyde, is then released while $PQQH_2$ is reoxidized by sequential single-electron transfer to a *c*-type cytochrome in the electron transport chain.

The mechanism of the oxidation for GDH is similar to the one described for methanol oxidation by MDH. The proton in the anomeric hydroxyl group of glucose

is abstracted by an aspartate residue in the active site of the enzyme, and a hydride is transferred from the C-1 of the glucose molecule to the C-5 carbon of the PQQ cofactor, yielding gluconolactonate and $PQQH_2$. Further oxidation of $PQQH_2$ involves either ubiquinone or *c*-type cytochromes as electron acceptors in the electron transport chain. The enzyme from *E. coli* is a monomeric protein of 87 kDa [191]. The *N*-terminal domain (154 residues) has five transmembrane segments, and it is likely where the ubiquinone-binding site is contained. The remaining periplasmic region (641 residues) is similar to the α-subunit of MDH. The prosthetic group PQQ in MDH is held in place in the active site by stacking interactions between a tryptophan residue and a disulfide ring structure made up of adjacent cysteine residues [192,193]. In GDH from *E. coli*, this disulfide ring is replaced by a histidine residue (His262), which is essential for binding PQQ [191]. The entrance to the active site is more open than in MDH, and His262 is located at the entrance, together with a second histidine residue (His775). GDH from *E. coli* has two ubiquinone binding sites: one of them is located near the active site and contains a tightly bound ubiquinone that performs single electron transfer steps; the second site is in the amphiphilic segment of the membrane bound GDH and reversibly binds ubiquinone from the membrane pool [194]. In the cell, the enzyme is responsible for the direct oxidation of glucose to gluconate in the periplasm, but also showed the ability to oxidize a wide range of pentoses and hexoses [191]. Disaccharides such as maltose, lactose, trehalose, and sucrose are neither substrates for GDH, nor was the trisaccharide raffinose, or D-fructose, *myo*-inositol, methanol, or ethanol. None of these compounds inhibited the oxidation of glucose, showing that they are not able to bind at the active site. L-Hexoses were not substrates, including L-glucose, L-mannose, L-allose and L-rhamnose. The only common feature of these L-hexoses is that they have a C-6 hydroxymethyl (or methyl) group below the plane of the ring, suggesting that this prevents binding by steric hindrance. As understanding of the mechanism of catalysis and electron transfer of quinoproteins progressed, the application of these enzymes as biosensors became attractive, and thus electrochemical studies aimed at the design of specific quinoprotein-based electrodes have increased [184]. Soluble glucose dehydrogenase (s-GDH) from *A. calcoaceticus* was the first quinoprotein applied to such a biosensor, and a D-glucose sensor with s-GDH in a single-use electrochemical test strip containing ferricyanide as a mediator is already in the market [184].

1.3.1.5 Dehydrogenases without Prosthetic Group Although most redox enzymes require metal or organic prosthetic groups for the hydride transfer, several dehydrogenases have neither metal ions nor any other prosthetic group bound to their active sites. The most prominent example of such enzymes is formate dehydrogenase (FDH), well known for its use in enzyme-coupled cofactor regeneration systems (see Chapters 2 and 8). The enzyme catalyzes the oxidation of formate to CO_2, cleaving a single carbon–hydrogen bond in the substrate and concurrently forming a new one in the nicotinamide cofactor, which also accepts the electrons [195]. During catalysis, a hydride anion from the substrate formate attacks the electrophilic C-4 of the nicotinamide cofactor NAD^+. Consequently, two uncharged products, CO_2 and NADH, are formed [144,196].

1.3.2 Oxygenases

Oxidative biotransformations with oxygenases are very valuable tools for synthetic organic chemistry. The most outstanding reason is that these enzymes catalyze a wide number of reactions including aliphatic and aromatic hydrocarbon hydroxylations, epoxidation of alkenes and arenes, *N*-, *S*-, and *O*-dealkylations, *N*-hydroxylations, *N*- and *S*-oxidations, deaminations, dehalogenations, and Baeyer–Villiger oxidations, with high regio- and stereoselectivity [35,149,197], while their chemical counterparts either do not exist or do not reach the required selectivity (see Chapter 4). In fact, many types of reactions that can be easily performed by oxygenases, such as asymmetric hydroxylations at saturated or unsaturated carbon atoms, or oxidative desymmetrizations, are difficult to carry out with conventional stoichiometric oxidants or by using organic or inorganic catalysts. Furthermore, when considering environmental aspects of many chemical oxidations, the replacement of hazardous procedures by more benign ones is highly desirable (see Section 1.1). The goal of reducing stoichiometric waste requires catalytic processes, and the quest for sustainable chemistry puts oxygenases in a central role for a variety of asymmetric oxidations [143,145,197–201]. Furthermore, oxygenases use molecular oxygen as a readily available and environment friendly oxygen source. Moreover, oxygenase-catalyzed reactions can be used to prepare highly valued building blocks and pharmaceutical intermediates, as well as to modify natural products with biological activities [197].

Oxygenase-catalyzed hydroxylations, namely the conversion of a C–H bond into a C–OH bond, are widespread enzymatic reactions occurring in all forms of life, from bacteria to mammals. Oxygenases are capable to insert one (monooxygenases) or two (dioxygenases) oxygen atoms from O_2 into their substrates under mild conditions [37,144]. In monooxygenase-catalyzed reactions, the second oxygen atom is reduced with electrons from NADH or NADPH, yielding water. This relatively complex reaction often requires a metal center, and electron transfer from reduced cofactors usually requires additional proteins. Dioxygenases catalyze the regio- and stereoselective insertion of both oxygen atoms from molecular oxygen into a substrate. Such highly specific oxyfunctionalizations, and especially the activation of C–H bonds, are synthetically interesting and often inaccessible by chemical means [202]. However, the use of oxygenases in organic synthesis is still limited due to their scarce availability in adequate quantities, their low stability and specific activity, and the high cost of required cofactors. Moreover, many of these enzymes are membrane-associated proteins, which reduce their potential application in synthetic procedures even more. Some of these drawbacks of oxygenases could be solved by the use of whole-cell biocatalysts, either as growing or resting cells, and preferentially recombinant microorganisms overexpressing the desired enzyme. Thus, presently the use of dioxygenases, such as arene dioxygenases, has mostly been described in whole-cell systems.

There are two major subclasses of oxygenases which incorporate dioxygen into their substrates: EC 1.13.- and EC 1.14.-. The enzymes of the former subclass do not need external hydrogen donors, such as NAD(P)H, for oxygenation and act on single substrate molecules, whereas those of the latter act on paired hydrogen donors [37]. Both, monooxygenases and dioxygenases are found in these subclasses.

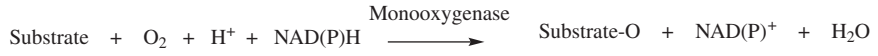

FIGURE 1.6 General scheme for monooxygenase-catalyzed reactions.

1.3.2.1 Monooxygenases Monooxygenases are enzymes capable of activating molecular oxygen and oxidizing a wide diversity of almost nonreactive organic compounds such as alkanes, alkenes, aromatic compounds, or heteroatoms [203–207]. These enzymes incorporate only one oxygen atom from molecular oxygen, while the other one is reduced to yield water. A broad spectrum of useful reactions can be developed with monooxygenases as biocatalysts, for example, hydroxylations, epoxidations, Baeyer–Villiger oxidations, oxidations of heteroatoms such as N and S, and dealkylations of heteroatoms [143]. The catalytic mechanism differs significantly depending on the enzyme subtype involved, but in all cases the activation of molecular oxygen takes place in a similar way. Molecular oxygen is activated by reduction at the expense of NADH or NADPH (donor). One oxygen atom is transferred to the substrate, and the other one is reduced to form one molecule of water (Figure 1.6).

Monooxygenases can be classified according to their prosthetic group in iron-containing monooxygenases (heme and nonheme iron monooxygenases), copper containing monooxygenases, flavin-dependent monooxygenases, multicenter monooxygenases, namely Fe-flavin monooxygenases (FMOs) and Fe-pterin monooxygenases, and cofactor-free monooxygenases [118,144].

Iron-containing Monooxygenases

NONHEME IRON-CONTAINING MONOOXYGENASES Nonheme iron-containing monooxygenases are mono- or binuclear enzymes which are able to catalyze the oxidation of aliphatic or aromatic hydrocarbons to the corresponding monohydroxylated compounds. Binuclear nonheme iron monooxygenases catalyze monooxygenations of alkyl-, benzyl- and aryl-nonactivated carbon atoms, as well as fatty acid desaturations and ribonucleotide reduction. Prominent examples of these enzymes are soluble methane monooxygenase (MMO), alkane monooxygenase (AMO), xylene monooxygenase (XMO), toluene-2-monooxygenase (T2MO), and toluene-4-monooxygenase (T4MO) [144,208–210].

The soluble MMO (s-MMO) is structurally and biochemically the best characterized member of this oxygenase family [210–213]. These enzymes are unique among the di-iron monooxygenases catalyzing the NADH-dependent insertion of one oxygen atom from O_2 into the exceptionally stable C–H bond of methane (bond dissociation energy of 104 kcal/mol) to form methanol, and typically accept a broad substrate spectrum. The soluble form of the enzyme (s-MMO) contains a 251 kDa dimeric hydroxylase protein (MMOH) with three subunits in a $(\alpha\beta\gamma)_2$ stoichiometry, together with a reductase (MMOR) and a regulatory "B" component (MMOB) [210,214–216].

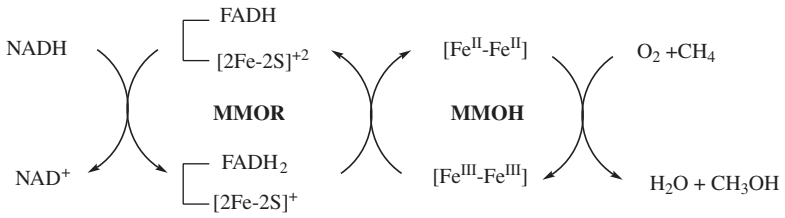

FIGURE 1.7 Catalytic mechanism of MMO.

Each subunit of MMOH contains a carboxylate- and a hydroxo-bridged dinuclear iron cluster, which is the site of catalysis, where dioxygen activation and methane hydroxylation occur. Typically, four glutamate and two histidine residues coordinate the di-iron center. The remaining of the coordination sites is occupied by solvent-derived ligands. MMOR is a 38.5 kDa iron–sulfur flavoprotein that shuttles electrons from NADH through its FAD and [2Fe–2S] cofactors to the hydroxylase active site. MMOB (15.9 kDa) does not contain prosthetic groups and modulates MMO reactivity by forming specific complexes with the hydroxylase that indirectly affect the structure and reactivity of the di-iron site (Figure 1.7) [208,215].

MMO is a classic monooxygenase in which two reducing equivalents from the cofactor are required to split the O–O bond of O_2. These reducing equivalents are used to form H_2O with one of the oxygen atoms, while the second is incorporated into methane with nearly 100% efficiency [211]. AMO and XMO show similar reaction mechanisms but differ from water-soluble proteins of the di-iron-carboxylate type in that their oxygenase component is membrane bound and the di-iron cluster is only complexed by histidines [217–219]. These enzymes contain a characteristic 8-histidine motif and a variable number of subunits. AMO consists of an integral membrane hydroxylase and two soluble proteins, rubredoxin and rubredoxin reductase, whereas XMO is a two- component enzyme, consisting of an integral membrane hydroxylase and a soluble NADH-acceptor reductase. Both enzymes accept a large substrate spectrum [144]. AMO, which catalyzes in nature the NADH-dependent hydroxylation of medium chain alkanes (C5–C12), accepts a wide range of linear, branched, and cyclic alkanes as well as alkyl benzenes [220,221]. Epoxidation of terminal alkenes, sulfoxidations and demethylations have also been reported [222–225]. XMO accepts *m*- and *p*-ethyl-, methoxy-, nitro-, and chloro-substituted toluenes, *m*-bromo-substituted toluenes and styrene, which is transformed into (*S*)-styrene oxide with high enantiomeric excesses [226]. The enzyme expressed in *E. coli* catalyzes the multistep oxidation of xylenes to the corresponding alcohols, aldehydes, and acids *via* individual monooxygenations [227,228].

Phenylalanine hydroxylase (PheOH, phenylalanine 4-monooxygenase, EC 1.14.16.1), tyrosine hydroxylase (tyrosine 3-monooxygenase, EC 1.14.16.2) and tryptophan hydroxylase (tryptophan 5-monooxygenase, EC 1.14.16.4) constitute a small family of monooxygenases that use tetrahydropterins as substrate [229]. Enzymes from eukaryotic sources are composed of a homologous catalytic domain attached to *N*-terminal regulatory domains and short *C*-terminal tetrameric domains, whereas the

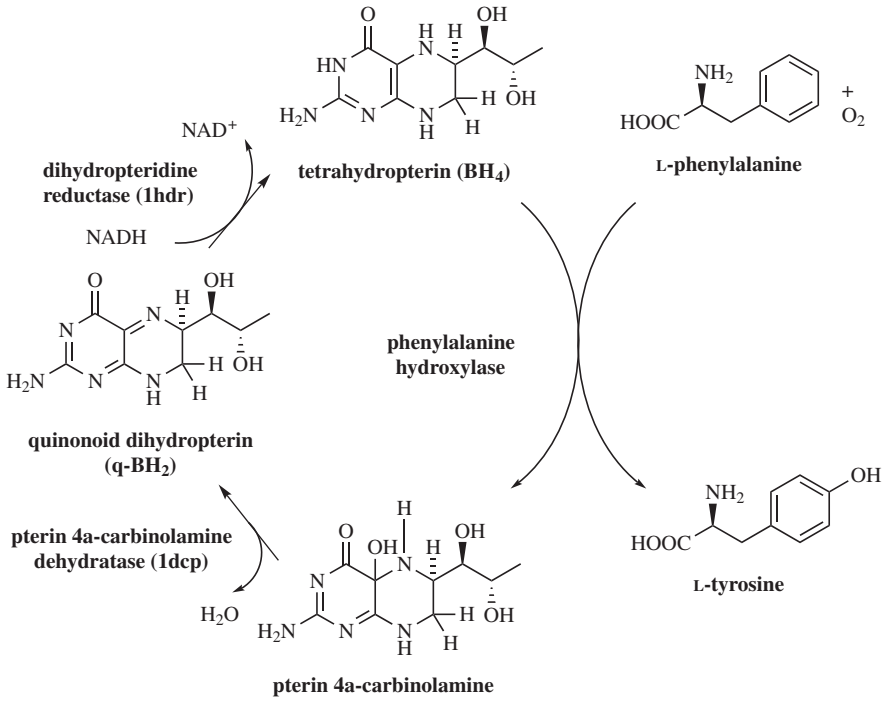

FIGURE 1.8 Reaction of phenylalanine hydroxylase (PheOH) and concomitant cofactor regeneration.

bacterial enzymes lack the *N*- and *C*-terminal domains. Each enzyme contains a single ferrous iron atom bound to two histidines and a glutamate. Although the hydroxylating intermediates in these enzymes have not been identified yet, the iron is likely to be involved. The mononuclear PheOH hydroxylates L-phenylalanine (L-Phe) into L-Tyrosine (L-Tyr) in the presence of (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH_4) as cofactor and dioxygen [230,231]. The enzyme is allosterically regulated by its substrates, phenylalanine and tetrahydrobiopterin. During the hydroxylation, the BH_4 cofactor undergoes a two-electron oxidation to a 4a-hydroxydihydrobiopterin (pterin 4a-carbinolamine), which is subsequently dehydrated to quinonoid dihydropterin (q-BH_2) by a carbinolamine dehydratase (EC 4.2.1.96), before being reduced by a dihydropteridine reductase (EC 1.6.99.7) (Figure 1.8) [232].

Structurally and functionally homologous to PheOH are TyrOH and TrpOH, all forming the family of the tetrahydrobiopterin-dependent aromatic aminoacid hydroxylases [232]. The three enzymes have a three-domain structure with an *N*-terminal regulatory domain (residues 1–142 in human PheOH), a central catalytic domain (residues 143–410 in human PheOH) that includes the active site-iron ligated to a 2-His-1-Glu facial triad [233,234], and a *C*-terminal tetrameric domain (residues 411–452 in human PheOH) [235–238]. The crystal structure of rat TyrOH with BH_2 has been reported, and the pterin was found to be located in the second coordination

sphere of the iron center [239]. The pterin binding site has been proposed to be in a highly conserved region in all aromatic amino acid hydroxylases [231].

HEME IRON MONOOXYGENASES CYTOCHROMES P450 Cytochromes P450 (P450s) are all heme-thiolate proteins whose prosthetic group is a protoheme (iron protoporphyrin IX) with a cysteine residue as the axial ligand [240]. With the exception of soluble bacterial and fungal P450s, the majority of these enzymes are membrane bound, associated with either the inner mitochondrial or the microsomal membranes [36]. While the number of reported nonheme monooxygenases is still limited, there are more than 10,000 notified genes for these proteins, divided in over 970 families [241] and widely distributed in almost all eukaryotes, prokaryotes, and even in hyperthermophilic archaea. With more than 500 isozymes, cytochromes P450 are widespread in nature. Molecular weights are typically about 40–60 kDa, and they all show high similitude in terms of global topology, position, and orientation of their characteristic secondary structures [242]. Although enzymes with less than 40% sequence identity are placed in different gene families, the tertiary structure in the active site for proteins of different families remains unchanged even at as low as 20% sequence identity, thus pointing to a common mechanism of oxygen activation [38,243]. Regarding the primary structure, only three amino acids are absolutely conserved in all P450 enzymes, playing a key role in the maintenance of the secondary and tertiary structures, as well as in catalysis [38,244]. A cysteine residue provides the iron heme of a thiolate ligand, which is essential for the catalytic activity and common to all P450s. In contrast, variable regions are represented by the flexible substrate recognition region (SRS) [245], which enable P450s to be highly versatile biocatalysts. This explains the remarkable variety of chemical reactions catalyzed and the wide range of structurally different substrates accepted by these enzymes. Each P450 has a preferred set of substrates ranging from steroids to polycyclic aromatic hydrocarbons to fatty acids. Therefore, each must have a unique method of substrate recognition and an active site allowing regio- and stereospecific monooxygenation, yet they all appear to have similar structural cores [38]. These enzymes are essential in primary and secondary metabolism, as well as in the detoxification of xenobiotics and drugs. In mammals, P450s are involved in several processes in steroidogenesis, mainly in the gonads and adrenal glands, with six different P450s functioning in the three steroid pathways, and in the arachidonic acid cascade, producing prostaglandins and epoxy- and hydroxy eicosanoic acids. Low concentrations of these enzymes are found in the brain and skin.

This type of monooxygenases catalyzes the hydroxylation of a wide variety of substrates, involving the transfer of molecular oxygen to –C–H, –N–H or –S–H bonds in aliphatic or aromatic nonactivated compounds, from simple benzene to complex ring systems such as flavonoids or aromatic steroids (see Chapter 4) [37,246,247]. Moreover, many P450 enzymes epoxidize C–C double bonds [248]. In addition to oxygen transfer, P450s are responsible for other chemical reactions including dealkylations, *S*- and *N*- oxidations, alcohol, and aldehyde oxidations as well as dehalogenation and denitration, which are performed with remarkable selectivity [249]. P450 enzyme-catalysis requires a redox partner to transfer electrons from

reduced nucleotides (NAD(P)H) to the enzyme for the binding and activation of the iron-bound molecular oxygen [37,38]. Some P450s do not require any other protein component for the reductive activation of molecular oxygen [250], but the vast majority of P450s performs the diverse range of chemical reactions after the interaction with one or more redox partners to provide their redox equivalents from electron transfer chains. Most of the electron transfer reactions begin with the transfer of electrons from NAD(P)H and end with the reductive cleavage of oxygen, with the concomitant incorporation of one oxygen atom into the substrate by the terminal P450 enzyme [251].

Although P450s are divided into gene families, they can also be classified into different groups, depending on the required redox partner. After the discovery of cytochromes P450, two main classes concerning the involved redox partners had been defined, the adrenal mitochondrial/bacterial type (Class I) [252], obtaining electrons from NADPH *via* adrenodoxin reductase and adrenodoxin, and the liver microsomal P450 (Class II) [253,254] obtaining electrons from NADPH *via* FAD and flavin mononucleotide (FMN)-containing P450 reductase [35,39,144,199,206, 255,256].

Class I: Most bacterial and mitochondrial eukaryotic enzymes belong to this class of P450 systems. The electron transfer system comprises three separated proteins: (i) a FAD-containing reductase, which transfers reduction equivalents from a pyridine nucleotide (NAD(P)H) to the second component of the system, (ii) a NAD(P)H ferredoxin reductase of the iron–sulfur [2Fe–2S] protein type, which shuttles electrons between the reductase, and (iii) the P450, reducing the cytochrome P450 itself [243]. In bacteria, all three proteins are soluble; in eukaryotes, only the ferredoxin is a soluble protein of the mitochondrial matrix, whereas the reductase and the P450 are membrane associated and membrane bound to the inner mitochondrial membrane, respectively [35,257]. Examples of Class I P450s are involved in the side-chain cleavage of cholesterol ($P450_{scc}$, CYP11A1, mammalian), the 11β-hydroxylation of 11-deoxycortisol, the production of aldosterone, in vitamin D biosynthesis, or in the 5-*exo*-hydroxylation of camphor (bacterial $P450_{cam}$, CYP101).

Class II: They occur often in eukaryotes and perform diverse reactions. In mammals they are responsible of the oxidative metabolism of endogenous compounds (fatty acid, steroids, prostaglandins) as well as exogenous compounds (therapeutic drugs, environmental toxics and carcinogens) [258]. Class II P450s from plant origin are involved in the secondary metabolism as well as in the synthesis of cutin and lignin barriers, and of defense substances [259]. In fungi, their activity includes the synthesis of membrane sterols and mycotoxins, and the detoxification of phytoalexins [243]. Microsomal cytochromes P450s are membrane-bound enzymes, and accept electrons from a microsomal NADPH-cytochrome P450 reductase (CPR), containing FAD and FMN. All drug- and xenobiotic-metabolizing cytochrome P450s, including CYP102 (P450(BM3)) isolated from *Bacillus megaterium*, as well as most of the other bacterial P450s belong to this class. These P450 systems consist on a polypeptide chain with two different domains, one containing the heme-protein and the other one containing an FAD-reductase. These soluble enzymes obtain the required electrons from an NADH-dependent FAD-containing reductase *via* an iron–sulfur protein

of the [2Fe–2S] type. While FAD serves as an electron acceptor from NADPH, the FMN moiety transfers the electrons to the oxygenase protein.

After the discovery of the first bacterial P450 system, the camphor hydroxylase [260,261], it was found that this system was organized analogously to the mitochondrial ones, transferring electrons from NADH *via* an FAD-containing reductase (putidaredoxin reductase) and an iron–sulfur protein ([2Fe–2S] type, putidaredoxin) to CYP101 ($P450_{cam}$) [243]. The discovery of CYP102 (P450(BM3)) in the 1980s showed an unusual property, a fusion of the P450 reductase and reductase components into one polypeptide chain [262,263]. Since then, several different electron transfer chains for diverse P450s have been described.

Class III: In 1998, Peterson et al. described Class III P450s as enzymes that do not require an exogenous source of electrons, as they employ either endoperoxide or hydroperoxide substrates, which already contain oxygen. The mammalian enzyme thromboxane synthase (CYP5A1) and the plant enzyme allene oxide synthase (CYP74) were included in this classification. However, more recently Bernhardt and coworkers described Class III P450s as enzymes with a novel redox partner system, which were described in 2002 [264], having high similarity to those of the classical bacterial system. These enzymes are also three-component P450 systems, in which electrons are transferred from the primary electron donor (NAD(P)H) *via* a NAD(P)H-dependent FAD-containing ferredoxin reductase, and a second auxiliary redox protein. An example is the novel cytochrome CYP176A1 (P450(cin)) from *Citrobacter braakii*, in which the intermediate electron donor of the cytochrome P450 is suggested to be a flavodoxin, instead of the iron–sulfur protein characteristic of the Class I systems. Thus, the electrons are delivered *via* the redox centers FAD and FMN, and not *via* FAD and iron–sulfur cluster, resembling the eukaryotic microsomal system, though the two redox centers FAD and FMN belong to separate proteins [264,265].

Class IV: A ferredoxin and its corresponding partner, 2-oxoacid:ferredoxin oxidoreductase constitute an efficient redox chain in this type of P450s. The soluble CYP119 (EC 1.14.14.-) [266,267] from the extremely acidothermophilic archaeon *Sulfolobus solfataricus* was the first example of a P450 enzyme that does not obtain its reducing equivalents from a NAD(P)H-dependent flavoprotein. More recently, another thermophilic cytochrome P450 has been found in *Sulfolobus tokodaii* [268], but apart from the enzyme structure, named P450(st), no other information is available so far [267].

Class V: This class of P450 enzymes consists of two separated protein components: a putative NAD(P)H-dependent reductase, and a cytochrome P450-ferredoxin fusion protein [243]. There is only one known example belonging to this novel class, and it is the sterol 14α-demethylase CYP51 (EC 1.14.13.70) from *Methylococcus capsulatus*, which shows a unique primary structural organization [269]. In this enzyme, a P450 heme-monooxygenase domain is fused at the *C*-terminus to a [3Fe–4S] type ferredoxin domain *via* an alanine-rich linker region, which is thought to act as a flexible hinge allowing interactions between the two domains [243].

Class VI: This class is composed of a putative NAD(P)H-dependent flavoprotein reductase and a flavodoxin-P450-fusion protein, thus standing somewhere in between

the P450(BM3) and P450(cin) systems, which principally use the same redox centers (FAD, FMN and heme) but differ in the number and characteristics of separate proteins comprising the system [243]. The first example found of this class VI P450s is the cytochrome P450-like gene from *Rhodococcus rhodochrous* strain 11Y (xp1A), in which the cytochrome P450 is fused to a flavodoxin domain at its *N*-terminus [270,271].

Class VII: In a unique type of structural organization, a cytochrome P450 is *C*-terminally fused to a reductase domain, a phthalate dioxygenase reductase domain, which is usually not associated with P450 systems [243]. The first reported enzyme of this class was the cytochrome CYP116B2 (P450(RhF)) from *Rhodococcus* sp. strain NCIMB 9784 [272], whose cytochrome P450 domain displays 55% homology to the class I CYP116 (P450(ThcB)) from *Rhodococcus erythropolis* NI86/21 [273]. In this protein, the *N*-terminal cytochrome P450 domain and the *C*-terminal reductase domain are separated only by a short linker region of 16 amino acids. In the case of P450(RhF) enzyme, three distinct functional parts can be identified: (i) a FMN-binding domain, (ii) a NADH-binding domain, and (iii) a [2Fe–2S] ferredoxin domain. The flavin cofactor of the reductase receives the electrons from the pyridine nucleotide, and in this system is FMN rather than FAD as in the other P450 systems [273].

Class VIII: This class contains P450 proteins fused to their eukaryotic-like diflavin reductase partner (CPR) in a single polypeptide chain and therefore are catalytically self-sufficient as monooxygenases. These enzymes have been found in various prokaryotes and lower eukaryotes [274]. The most deeply studied enzyme of this class is the cytosolic fatty acid hydroxylase flavocytochrome CYP102A1 (P450(BM3), EC 1.14.14.1) from *B. megaterium* [243,275]. This was the first P450 discovered as a fusion (*N*-terminal) to its NADPH-CPR (EC 1.6.2.4). The enzyme is a soluble single polypeptide with a molecular weight of 119 kDa [262,276,277], composed of a heme-containing P450 oxygenase domain connected *via* a short protein linker to a diflavin reductase domain, containing one equivalent each of the cofactors FAD and FMN [262]. The fusion of the heme domain in CYP102A1 to its diflavin reductase in a single polypeptide is an arrangement similar to that of the nitric oxide synthases [263]. CYP102A1 catalyzes the NADPH-dependent hydroxylation of medium and long-chain saturated fatty acids at ω-1, ω-2, and ω-3 positions [263,278] displaying high monooxygenase activity [279,280]. NADPH reduces the protein with electrons transferred to the FAD cofactor in the CPR domain, and subsequently they are transferred one by one from the FAD to FMN and further on to the heme iron in the substrate-bound P450 domain [278,281]. The self-sufficient CYP505A1 (P450(foxy), EC 1.14.14.1) from *Fusarium oxysporum* displays an eukaryotic counterpart of the bacterial CYP102 [282]. The protein is a fusion of a P450 and a CPR domain on one gene, and is produced as a single polypeptide [283].

Class IX (so-called Class IV according to Peterson's classification) [38]: There is only one known example belonging to this group of P450s. Nitric oxide reductase, $P450_{nor}$ (CYP55A) receives its electrons directly from reduced pyridine nucleotides without the need of an electron carrier. Among heme-thiolate proteins, $P450_{nor}$ of the

filamentous fungus *F. oxysporum* was identified as a P450 with particular features [243]. The enzyme is located in the mitochondrial and cytosolic fractions of the cells, and represents the first and so far only soluble eukaryote P450 protein [284,285]. Its physiological role is part of the denitrification reaction, protecting the fungus from NO inhibition of mitochondria, especially when oxygen becomes limiting [286]. $P450_{nor}$ uses NADH as electron donor, and catalyzes, independent of other electron transfer proteins, the conversion of two molecules of nitric oxide to nitrous oxide as a P450s unique reductive process [287].

Class X: Most of P450 enzymes behave as monooxygenases, requiring a consecutive delivery of two electrons *via* different types of redox proteins. However, Class X P450 systems catalyze substrate conversion using an independent intramolecular transfer system. These enzymes include the independent cytochrome P450 allene oxide synthase (CYP74A, AOS), fatty acid hydroperoxide lyase (CYP74B/C, HPL), divinyl ether synthase (CYP74D, DES) (EC 4.2.1.92), prostacyclin synthase (EC 5.3.99.4), and thromboxane synthase (EC 5.3.99.5) [243]. AOS, HPL, and DES, located in membranes of chloroplasts, are key enzymes of plant LOX pathway, and are relatively atypical members of the cytochrome P450 family CYP74, which have not been detected in mammals [288]. These enzymes do not require O_2 or an NADPH-dependent CPR, or even the electron source NAD(P)H, for the rearrangements of fatty acid hydroperoxides [289–291]. They use the acyl hydroperoxide of the substrate as oxygen donor, thus forming new carbon–oxygen bonds in the products.

Regarding structural features of cytochromes P450, in the past years significant progresses have been made in the determination of the active-site structure of these enzymes [39]. The information has been obtained through the crystal structures of mammalian P450s [292–298], and complemented with the information available on bacterial and fungal cytochromes. The structural core of P450s is conserved, and formed by a four-helix bundle composed of three parallel helices labeled D, L and I and one antiparallel helix E [299]. The prosthetic heme group is located between the distal I helix and the proximal L helix and bound to an adjacent Cys-heme-ligand loop. This cysteine residue is absolutely conserved and is the proximal of "fifth" ligand to the heme iron, forming two hydrogen bonds with neighboring backbone amides. A further interaction is observed in some P450s with a Gln residue, and an example is CYP152A1. Mutations of these and similarly placed residues, for example, Phe393 in CYP102 (P450(BM3)), have pronounced effects on the reduction potential of the catalytic activity and on the stability of the bond between the heme iron and its ligands [39]. A highly conserved Thr residue located in the long I helix in the heme pocket preceded by an acidic residue is positioned in the active site and believed to be involved in catalysis [300,301].

The basic concepts of the catalytic cycle of P450s were developed in the early 1970s from the discovery of the role of these monooxygenases in steroid biosynthesis, and outstanding progress in the detailed understanding of the catalytic mechanism has been made in the past decade [39]. The use of a wide set of methodologies including systematic directed mutagenesis, high-resolution X-ray crystal structure determination, spectroscopic characterization of intermediates, isolation of critical steps using cryogenic or fast kinetic techniques, and computational studies gave

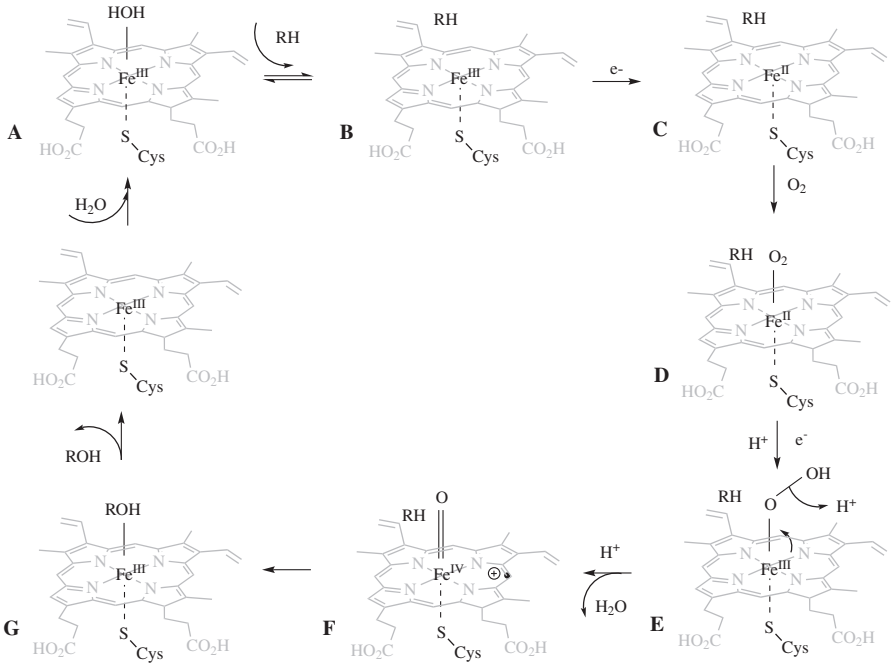

FIGURE 1.9 Cytochrome P450 catalytic cycle. The cysteine thiolate provided by the protein is represented as an S. RH, Substrate hydrocarbon; ROH, Resulting hydroxylated product. The ⊕• over the heme indicates that the radical cation is located on the porphyrins [240,302].

valuable information on the mechanism and species involved in the catalysis. The catalytic mechanism of P450 may be studied focusing on the states of heme iron and oxygen (see Figure 1.9) [240,302]. The main steps are (i) oxygen binding to the reduced heme iron and formation of an oxygenated heme Fe^{2+}–OO or Fe^{3+}–OO^-; (ii) reduction of this complex to a ferric peroxo state Fe^{3+}–OO^{-2}, which is easily protonated to form the hydroperoxo Fe^{3+}–OOH^-; (iii) a second protonation of the latter Fe^{3+}–OOH^- complex at the distal oxygen atom to form an unstable transient Fe-OOH_2, followed by the heterolytic scission of the O–O bond and release of a water molecule; and (iv) the reactions of the remaining higher valent porphyrin metal-oxo complex, named as a ferryl-oxo π-cation porphyrin radical [39]. Thus, the catalytic cycle of P450s starts with the resting enzyme in the ferric state, having a thiolate proximal ligand, which is usually a water molecule (**A,** Figure 1.9) [303], despite a few P450 enzymes do not have a ligand coordinated to the iron ion on the distal side (e.g., the case of human CP2D6) [304]. Substrate binding causes the displacement of the water ligand, resulting in a shift in the redox potential of the heme iron atom by up to 300 mV, enabling the electron transfer from the redox partner (**B**) [240]. The ferric cytochrome P450 is reduced by an associated reductase with an NADPH-derived electron to the ferrous cytochrome P450 (**C**). The formed ferrous substrate-bound protein then binds to molecular oxygen to yield the ferrous

cytochrome P450-dioxygen complex (**D**). A second electron transfer generates the ferric peroxy anion, which is protonated to give the ferric hydroperoxo complex (**E**). This second electron transfer is usually (with exceptions) [305] the rate-limiting step of the catalytic cycle. The ferric hydroperoxo intermediate is unstable and upon protonation, gives a porphyrin radical cation Fe(IV) as intermediate (**F**) so-called "compound I," or, as it was alternatively proposed, an Fe(V)=O species [35]. This ferryl intermediate is two oxidation states above the resting ferric state. It attacks the substrate to produce the hydroxylated metabolite (**G**) and, after product release and equilibration with water, the resting ferric state of the enzyme is achieved to start the cycle again. The ferric peroxy anion and the ferric hydroperoxo complexes could be observed by spectroscopic and electron paramagnetic resonance (EPR) methods [306–310], showing that the oxygen of the hydroxylated compound is coordinated to the heme iron atom [311].

The interaction of the P450s with their corresponding electron donor is necessary for the catalysis. Its specificity assures a sufficient catalytic rate and discrimination among different potential donors and acceptors of electrons to protect the system from shunt reactions [35]. Since in liver microsomes many different isoenzymes interact with only one reductase type, it is expected that the binding site for reductase is similar in various cytochrome P450s. The electrons for the reduction of the heme iron atom to the ferrous state in the P450s catalytic cycle are provided—depending on the specific enzyme—by (i) CPR, which is a membrane-bound protein containing a FAD and a FMN as prosthetic groups in enzymes such as CYP3A4 and CYP2C9; (ii) an iron–sulfur protein which shuttles electrons from a flavoprotein with a single FMN prosthetic group, and this is the case of CYP101 and CYP11A1; or (iii) a P450 reductase-like domain fused to the P450 heme domain within a single polypeptide, such as in CYP102 [243,244,312]. Isolated examples of other electron donor partners such as flavodoxin [264], a 2-oxoacid ferredoxin oxidoreductase [313], and a fusion protein which incorporates both, a ferredoxin and a ferredoxin reductase into a single polypeptide with the P450 enzyme have also been reported [314]. Salt bridges are responsible for the recognition of the reductase by the P450, and for the correct orientation of both proteins to each other [257].

In mitochondrial steroid hydroxylases such as the camphor hydroxylating bacterial $P450_{cam}$ (CYP101) systems, a charge-pair interaction mechanism has been demonstrated by chemical modification, site-directed mutagenesis, and structural data of electron-transfer complexes [35,315]. Similar to microsomal reductases, the mitochondrial ferredoxin has also the function to deliver electrons to different cytochromes P450. A shuttle model is favored, where the oxidized ferredoxin interacts first with the ferredoxin reductase and further undergoes the reduction with the corresponding formation of an Fe^{3+}/Fe^{2+} iron–sulfur cluster. This cluster is then dissociated from the reductase, and interacts with the respective P450, where it delivers this electron before returning to the reductase and the second electron is transferred to the P450. However, the mechanism of electron transfer between the components of different cytochromes P450 is not well understood yet [35]. It has been suggested that posttranslational modifications can regulate these electron transfer reactions [316]. This electron transfer to the P450 seems to be low and rate limiting

in P450 catalysis, therefore, engineering of this step leads to significantly improved biocatalysts [317].

Flavin-Dependent Monooxygenases Flavin monooxygenases (FMOs, EC 1.14.13.-) catalyze a wide variety of biochemical processes, including microbial biodegradation of activated aromatic compounds such as phenols, salicylic acid, or *p*-hydroxybenzoic acid, the oxygenation of nitrogen-, sulfur-, phosphorous-, selenium-, and other nucleophilic heteroatoms-containing chemicals, including many drugs, and the Baeyer–Villiger reaction [318]. Depending on the type of enzyme, flavin is either tightly but noncovalently bound and reduced in the monooxygenase moiety itself, or acts as an electron shuttle being reduced by a separate reductase component, and then bound and stabilized by the monooxygenase component. In any case, the electrons are derived from NAD(P)H, thus involving a direct coupling to the redox metabolism of living cells [144]. The overall reaction catalyzed by FMOs involves three general chemical processes: (i) the reduction of the flavin by NAD(P)H; (ii) the reaction of the reduced flavin with O_2 to provide a C-4a-flavin (hydro)peroxide which is the oxygenating agent (the peroxide for electrophilic substrates and the hydroperoxide for nucleophilic substrates); and (iii) binding, orienting, and activating of the substrate for its oxygenation by the C-4a-(hydro)peroxide (Figure 1.10) [145,318,319]. These three processes have unique requirements and usually more than one active site in the enzyme is required.

In single-component FMOs, such as *p*-hydroxybenzoic acid hydroxylase (PHBH, EC 1.14.13.2), there is an open conformation that gives access of both substrates and products to solvent, and a closed conformation for the reaction with oxygen and where the hydroxylation occurs. This closed form prevents from the destabilization of the (hydro)peroxy-flavin intermediate by the solvent. Finally, the so-called "out conformation," is achieved by movement of several angstroms of the isoalloxazine ring of the flavin toward the solvent, which exposes its N-5 for hydride delivery from

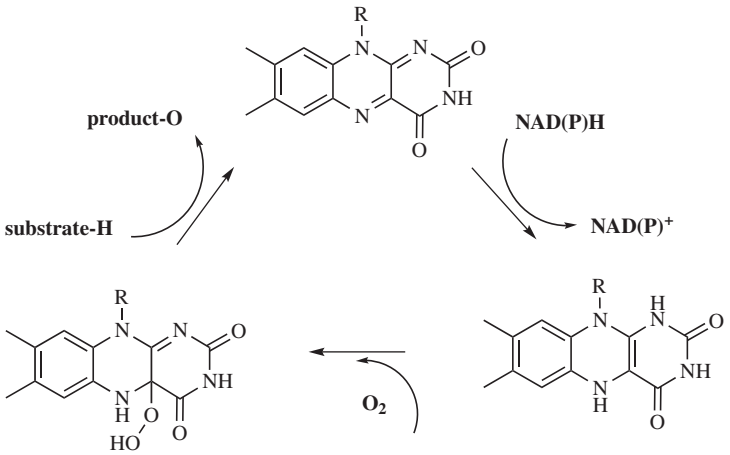

FIGURE 1.10 Schematic oxygenation mechanism for flavin-dependent monooxygenases.

NAD(P)H. The protein undergoes significant rearrangements during the catalysis. This group of enzymes have multiple active sites [318,320–326]. The enzymes PHBH and phenol hydroxylase (EC 1.14.13.7) play important roles in soil detoxification processes, and can be found in prokaryotic microorganisms (*Pseudomonas putida*) as well as in eukaryotes (*Trichosporon cutaneum*, human liver). They are capable to introduce a second hydroxyl group in *ortho-* position with respect to the previously existent one [318,325,327–329].

The group of two-component hydroxylases is characterized by two separated catalytic roles. These enzymes use an oxidoreductase to generate the reduced flavin, and an oxygenase that receives the reduced flavin and reacts with O_2 hydroxylating the substrate. These two-component systems transfer the reduced flavin from the reductase to the oxygenase, stabilizing a C-4a-peroxyflavin until the substrate binds to be hydroxylated, all before flavin oxidation and release of H_2O_2. The two-component systems have no structural or sequence similarities to the one-component enzymes. The mechanisms through which the labile reduced flavin is transferred from the reductase to the oxygenase in the two-component systems is still not well understood, although it is suggested that for most, the reduced flavin diffuses to the oxygenase components before it reacts with oxygen, so that the appropriate hydroxylating species are formed without the production of H_2O_2 [318,330,331].

For all these enzymes, flavin is reduced by NAD(P)H for its reaction with O_2. This reduction occurs stereospecifically by hydride transfer, usually with the *pro-R* hydrogen of the reduced pyridine nucleotide being transferred to the N-5 of the flavin isoalloxazine [332]. The reduction of flavin is often a critical control point in flavoprotein monooxygenase-catalyzed processes [318]. For most of the known single-component flavoprotein aromatic hydroxylases, the reduction is not effective in the absence of substrate, thus preventing the wasteful use of NAD(P)H that would produce reactive oxygen species such as H_2O_2. On the other hand, controlling the rate in the reduction does not regulate the Baeyer–Villiger oxygenases, or mammalian FMOs [333–335]. In these cases, NADPH reduces the enzymes at the same rate in the presence or absence of substrates, and the $NADP^+$ product remains tightly bound. The reduced enzyme-bound flavin then reacts with oxygen to form the C-4a-(hydro)peroxy-flavin, which is stable in the absence of substrates, and the bound $NADP^+$ stabilizes the intermediate. In the presence of substrate, the reaction with the C-4a-(hydro)peroxy-flavin is fast, yielding the oxygenated product [336,337]. Thus, turnover to produce H_2O_2 and oxidized flavin is avoided by a completely different mechanism than in the single-component aromatic hydroxylases. Within this group, Baeyer–Villiger monooxygenases (BVMOs) are NAD(P)H-dependent flavoproteins, which incorporate one atom of molecular oxygen into the substrate, and the other atom is reduced to water. These enzymes can be classified in two groups: type I BVMOs and type II BVMOs. Type I BVMOs are composed of a single chain protein containing FAD as cofactor, and use NADPH as source for electrons. A fingerprint motif was found in characterized type I BVMOs. The most outstanding example of this type of enzymes is cyclohexanone monooxygenase (CHMO, EC 1.14.13.22) from *A. calcoaceticus* NCIMB 9871, which was purified and characterized by Trudgill et al. [338]. The enzyme catalyzes the transformation of cyclohexanone into ε-caprolactone, through

the participation of an FAD-4a-hydroperoxyde intermediate, which is the nucleophile that reacts with the carbonyl group [339]. The enzyme has a broad substrate specificity accepting cyclic ketones as well as open chain ketones, aldehydes, and boronic acids (see Chapter 4) [327]. On the other hand, type II BVMOs contain FMN as cofactor, use NADH as electron donor, and are composed of $\alpha_2\beta$ trimers [340]. Examples of this type of enzymes are 2,5-diketocamphane monooxygenase (2,5-DKCMO) and 3,6-DKCMO (3,6-DKCMO) from *P. putida* ATCC 17453 (NCIMB 10007) (see Chapter 4). All BVMOs cloned could be classified as type I enzymes, while no type II BVMO sequence is known. There are no crystal structures available of BVMOs that would disclose the structural features of this class of enzymes [341].

Most of biochemical and mechanistic studies have been performed with type I BVMO [341,342]. The generally accepted mechanism for Baeyer–Villiger oxidation catalyzed by BVMOs was proposed by Walsh and coworkers based on results obtained with CHMO (Figure 1.11) [200,343].

First, the reduced cofactor NADPH binds to the enzyme and the tightly bound FAD prosthetic group (**A**) is reduced to $FADH_2$ (**B**). The cofactor is held in the active site in the oxidized form ($NADP^+$) until the last step of the catalytic cycle [200]. In fact, the release of the oxidized cofactor is the rate limiting step of the catalysis [333]. In the next step, this complex (enzyme-$FADH_2$-$NADP^+$) reacts with molecular oxygen to form the flavin-peroxide species (**C**), equivalent to peracids used in the chemical Baeyer–Villiger oxidation. The C-4a-peroxiflavin intermediate **C** is in equilibrium with the corresponding hydroperoxide **D** as demonstrated by spectroscopic studies [333,344]. It has been proposed that these two forms account for the ambivalent character of the BVMOs in catalyzing both, electrophilic (e.g., sulfoxidation of thioethers) and nucleophilic (e.g., Baeyer–Villiger reaction and epoxidation of alkenes) oxidation processes [344–346]. Attack of C-4a-peroxiflavin intermediate **C** at the carbonyl functional group of the ketone substrate produces a tetrahedral "Criegee-like" intermediate (**E**). This intermediate rearranges to give the C-4a-hydroxyflavin **F** and the corresponding ester or lactone (ε-caprolactone when cyclohexanone is the substrate). Water is spontaneously eliminated from the hydroxy-flavin generating the oxidized prosthetic group FAD (**A**). The catalytic cycle is finally closed by $NADP^+$ release. As in the chemical reaction, the enzyme-catalyzed reaction proceeds with retention of configuration at the migrating carbon atom, as determined by Schwab and coworkers employing isotopically labeled substrates [347,348]. Also, the same stereoelectronic effects (primary and secondary) drive the migratory preference, as long as only one C–C bond is antiperiplanar to the O–O bond in the tetrahedral intermediate **E** [342,349].

Copper-Containing Monooxygenases Copper is a key cofactor in a diverse array of enzymes involved in biological oxidation–reduction reactions [350]. Among monooxygenases, tyrosinase (PPO, EC 1.14.18.1), peptidylglycine α-amidating monooxygenase (PAM), and dopamine β-monooxygenase (DBM, EC 1.14.17.1) are outstanding examples of copper-containing enzymes.

Many bioactive peptides need to be amidated at their carbonyl terminus to exhibit activity, but these amides are not generated through transamidation reactions.

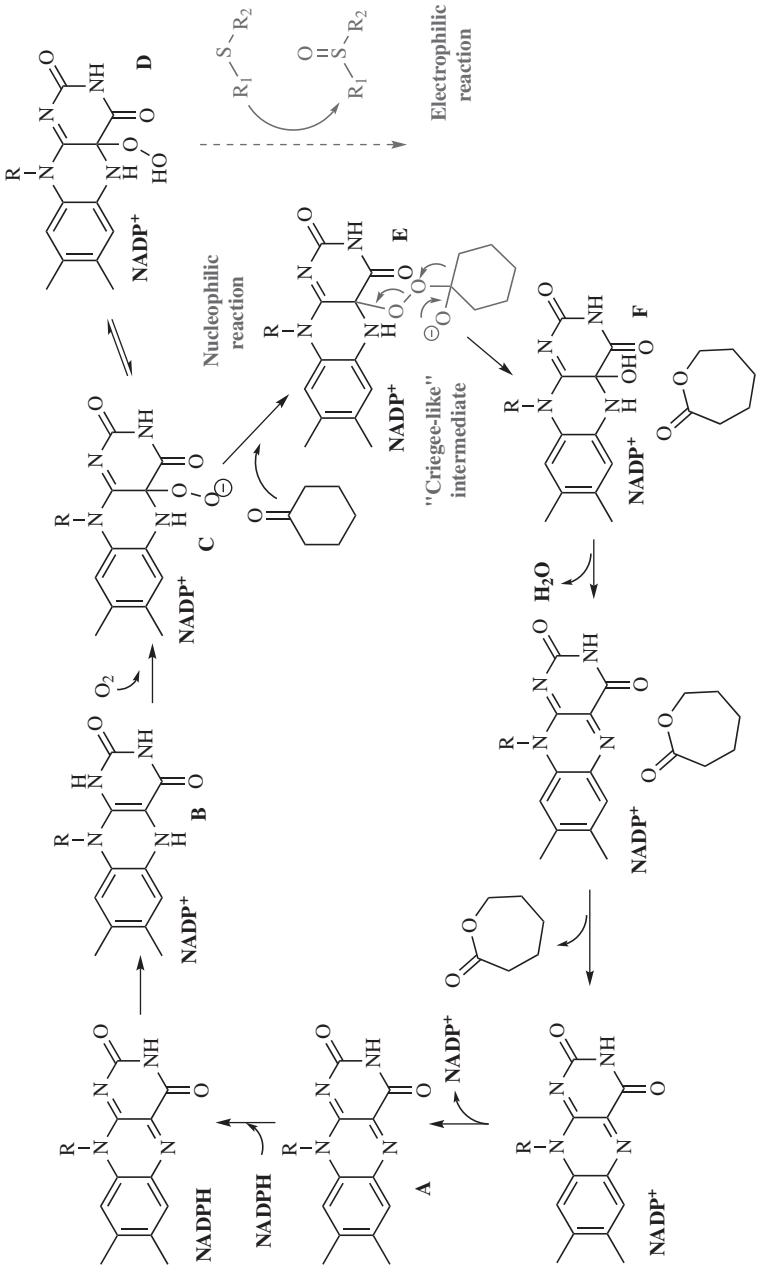

FIGURE 1.11 Proposed mechanism for the Baeyer–Villiger oxidation of cyclohexanone catalyzed with BVMOs of type I.

Instead, these amides are synthesized from glycine-extended intermediates that are converted into the active terminal amides by oxidative cleavage of the glycine N–Cα bond [351]. Although in some organisms this activity is developed by independently expressed enzymes, in higher organisms the process is catalyzed by PAM, a bifunctional copper-containing enzyme [351]. The PAM gene encodes one polypeptide with two enzymes catalyzing the two sequential reactions required for amidation, namely peptidylglycine α-hydroxylating monooxygenase (PHM, EC 1.14.17.3) and peptidylhydroxyglycine α-amidating lyase (PAL, EC 4.3.2.5). The former catalyzes the stereospecific hydroxylation of the glycine α-carbon of all the peptidylglycine substrates and the latter generates the α-amidated peptide product and glyoxylate. PHM is an ascorbate-dependent monooxygenase, containing two redox-active copper atoms that cycle between Cu^+ and Cu^{2+} during the monooxygenase reaction [352–354]. The catalytic cycle starts with the oxidized resting form of PHM, and the reduction of the two copper atoms from Cu^{2+} to Cu^+ by two one-electron transfers from two ascorbate molecules, which are oxidized to two semidehydroascorbate species, and then converted into one ascorbate and one dehydroascorbate molecule, producing the catalytically active PHM [354]. Then, the ternary complex of reduced enzyme with peptidylglycine substrate and molecular oxygen is responsible for the reaction progress through an alkyl radical intermediate [355,356], producing the amidated product, water, and the oxidized enzyme [351]. The reaction is stereospecific, involving the abstraction of the *pro-S* hydrogen, thus yielding the (*S*)-configurated product [357,358]. Molecular oxygen is then incorporated into peptidyl-α-hydroxyglycine product [359–361]. PHM is homologous in sequence and mechanism to another copper-containing monooxygenase, dopamine β-monooxygenase (DBM, EC 1.14.17.1), which converts dopamine into norepinephrine during catecholamine biosynthesis, thus the structural and mechanistic insights of PHM can be extended to DBM [351,362].

For these enzymes, two alternative catalytic mechanisms have been proposed differing in the nature of the hydrogen-abstracting species [363,364]. In both schemes, molecular oxygen binds to one of the copper atoms after ascorbate reduces the catalytic coppers. In the presence of substrate, each copper atom donates one electron to molecular oxygen, which undergoes a two-electron reduction to peroxide. This peroxide is protonated, forming a copper-bound hydroperoxide [365]. From this point, the two reaction schemes diverge. In one of the proposed approaches, homolysis of the hydroperoxide is coupled with the abstraction of hydrogen from the substrate [363]. The second approach relies on an active-site residue, which is supposed to be a tyrosine residue, donating a hydrogen atom to form a copper-bound radical [364]. Both mechanisms result in a copper-alkoxide complex, which dissociates to yield the free enzyme and the product.

Certain oxygenases are usually recognized separately from monooxygenases, for example, copper-containing oxidases [149,366]. One of these enzymes is a PPO – also known as tyrosinase or monophenol monooxygenase (EC 1.14.18.1), which is a monooxygenase that uses molecular oxygen as the electron acceptor in the site-specific hydroxylations and oxidations of phenols and catechols. Tyrosinase is a copper monooxygenase that belongs to a larger group or proteins named type 3

copper proteins [367–369]. Together with laccases (EC 1.10.3.2), tyrosinases belong to the group of PPOs, and are able to catalyze the oxidation of aromatic compounds by oxygen. Tyrosinase catalyzes both the *o*-hydroxylation of monophenols (cresolase activity) and the oxidation of *o*-diphenols to the corresponding *o*-quinones (catechol oxidase activity, EC 1.10.3.1) [350,370–372]. The enzyme is widely distributed throughout the phylogenetic scale including bacteria, fungi, plants, and mammals, and usually presents different characteristics in different organs of the same organisms, such as in roots and leaves of higher plants [373]. These enzymes are involved in the melanin pathway and are specifically responsible for the first steps of melanin biosynthesis, which involves the oxidation of L-tyrosine yielding L-3,4-dihydroxyphenylalanine (L-DOPA), and leading to the formation of L-dopaquinone and L-dopachrome [374]. The *o*-quinone products thus produced are spontaneously converted into melanin pigments *via* nonenzymatic reaction pathways [375]. The peculiarity of tyrosinase is that it catalyzes the *o*-hydroxylation of monophenols displaying monooxygenase activity, and also the subsequent oxidation of the resulting *o*-diphenols into reactive *o*-quinones, showing their catalytic oxidase activity [368]. Both reactions proceed using molecular oxygen.

Similar to other PPO, like catechol oxidase (EC 1.10.3.1), which is a ubiquitous plant enzyme catalyzing the oxidation of a broad range of catechols to the corresponding *o*-quinones, and hemocyanin, acting as an oxygen carrier and storage protein in mollusks and arthropods—tyrosinase has a coupled dinuclear active site [367,370]. Despite large differences in primary, tertiary, and quaternary structure, the oxygen binding-site is highly conserved in all these enzymes, suggesting that these proteins have evolved from the same origin. Basically, the catalytic center consists of a hydrophobic pocket inside a helix bundle comprising four densely pocket helices [376]. The structure is maintained by electrostatic and cation-π interactions among the helical segments. Two histidine-rich regions named CuA and CuB are the peptidic segment involved in the two coppers. Both regions contain three perfectly conserved histidine residues [370], and oxygen binds as a side-on peroxide bridge [376]. The catalytic mechanism of tyrosinase has been object of intensive research because of the complexity and peculiarities of the enzyme, such as the existence of two catalytic activities at the same active site and a lag period displayed by the cresolase activity which depends on the presence of catechols—the product of this reaction and the substrate for the oxidase activity [377]. The most accepted proposed reaction mechanism describes a common catalytic site for the two activities with three different forms of the enzyme, called, *met*-, *oxy*-, and *deoxy*-forms, according to the absence or presence of oxygen and the oxidation state of the copper ions [Cu(II)/Cu(I)] [368,372]. The *oxy*-form of tyrosinase and the appropriate substrates initiate either the oxidase- or the hydroxylase-cycles. Native tyrosinase occurs in the inactive *met*-form in which the binuclear copper site is in an inadequate oxidation state [Cu(II)] to bind dioxygen [378]. A two-electron reduction by a catechol converts *met*-tyrosinase into *deoxy*-tyrosinase, which readily binds dioxygen giving *oxy*-tyrosinase (Figure 1.12) [370].

In the *oxy*-form, one dioxygen molecule coordinates between the coppers, each of which is ligated to the protein matrix by three histidine residues provided by an

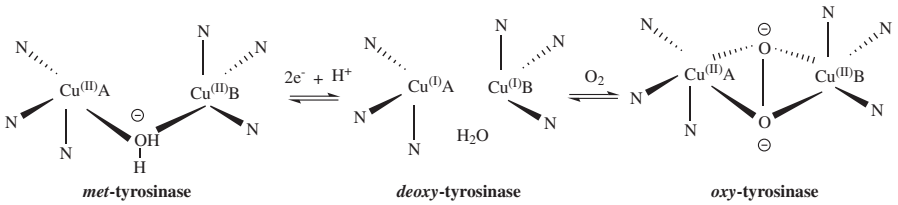

FIGURE 1.12 Binuclear copper-site in the catalytic mechanism of tyrosinase.

antiparallel α-helix pair, except for one His at the CuA motif that comes from a flexible loop. This histidine residue has been proposed as the residue involved in the proton shift from the *ortho*- position of the substrate tyrosine during the catalysis [379]. Oxygen binding induces an oxidative change in the valence of the copper ions that are at the Cu(I) state in the *deoxy*-form and become Cu(II) upon oxygenation. Both phenols and catechols are subsequently oxidized to *o*-quinones by *oxy*-tyrosinase, but the mechanisms of these oxidations are different [370,380]. Oxidation of catechols leads to *met*-tyrosinase, which cannot bind oxygen to regenerate *oxy*-tyrosinase. Only in the presence of a second catechol molecule the *met*-tyrosinase is reduced to *deoxy*-tyrosinase, which is able to regenerate the *oxy*-form (Figure 1.13).

Oxidation of monophenols by *oxy*-tyrosinase gives as final product an *o*-quinone and *deoxy*-tyrosinase, which binds further oxygen, and the oxidation cycle continues to completion (Figure 1.14) [370].

While all enzyme forms oxidize catechols to *o*-quinones, *met*-tyrosinase is inert toward phenols, though they bind reversibly to the active site. Therefore, reaction of phenols with native tyrosinase does not occur unless a catechol (added or formed by an indirect mechanism) leads to *deoxy*-tyrosinase formation. This aspect of phenol oxidation is responsible for the characteristic "lag period" observed in *in vitro* tyrosinase oxidations.

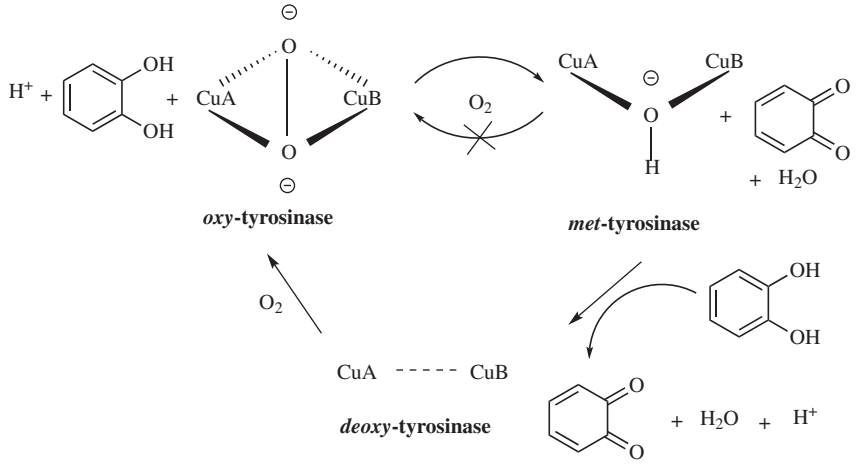

FIGURE 1.13 Catecholase cycle of tyrosinase.

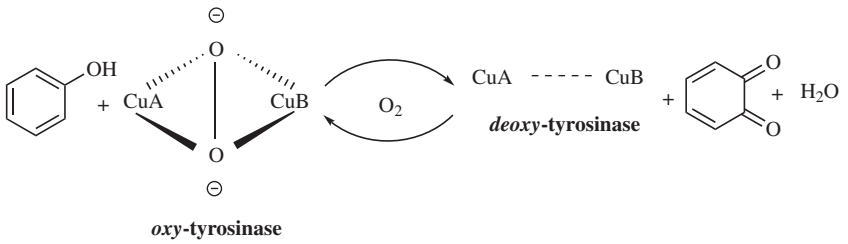

FIGURE 1.14 Cresolase cycle of tyrosinase.

Cofactor-Free Monooxygenases Although the majority of oxygenases require transition metal ions and/or organic cofactors, several enzymes undergo hydroxylations without the apparent need of cofactors [381,382]. Herein, a mechanistically intriguing problem is how triplet dioxygen is activated to react with a singlet organic compound. Among bacterial monooxygenases, quinone-forming monooxygenases involved in the tailoring of polyketides and luciferase monooxygenase from *Renilla* sp. (RLuc) have been identified as cofactor-independent enzymes [382]. Cofactor-independent monooxygenases catalyze very different reactions and belong to several different protein families, reflecting their diverse origin. Nevertheless, they all share the common mechanistic concept of initial base-catalyzed activation of their substrates and "substrate-assisted catalysis." *Renilla* luciferase possesses an α/β-hydrolase architecture for oxygenation reactions. The enzyme catalyzes the monooxygenation of coelenterazine to coelenteramide and CO_2, emitting blue light [382].

The biosynthesis of polyketides involves reactions catalyzed by polyketide synthases, which polymerize simple fatty acids to a polyketide chain, mediate in cyclization steps, and further reactions catalyzed by so-called tailoring enzymes. Type I polyketide synthases are multifunctional proteins that contain multiple active sites and are responsible for one complete cycle of building and modifying of the polyketide backbone. Type II polyketide synthases are constituted of monofunctional proteins that act iteratively [383–386]. Tailoring enzymes comprise reductases, methyltransferases, glycosyltransferases, decarboxylases, and different type of oxygenases. An important tailoring step in the synthesis of a number of aromatic polyketides is the oxidation of naphthacenone- and anthrone-type precursors of aromatic polyketides to the corresponding quinone derivatives. Functional studies on quinone-forming monooxygenases are available since 1990s, when tetracenomycin F1 monooxygenase (TcmH) was isolated from *Streptomyces glaucescens* by Hutchinson and colleagues [387]. The enzyme is a 12.6-kDa protein catalyzing the oxidation of the naphthacenone tetracenomycin F1 at C-5 to 5,12-naphthacenequinone tetracenomycin D3. This quinone-forming monooxygenase requires only dioxygen for activity and uses its substrate as reducing equivalent for the reduction of one oxygen atom of dioxygen to water. It does not possess either prosthetic groups known for monooxygenases, or contain or require metal ions for its catalytic process, albeit cofactors such as flavins or transition metal ions are thought to be required for the "activation" of molecular oxygen or the substrate [381]. It was suggested that sulfhydryl groups and histidine

residues could be essential for catalysis. A histidine residue has been proposed to act as a general base, allowing the dehydration of the proposed semiquinone peroxide intermediate [387]. The next quinone-forming monooxygenase to be isolated was ActVA-Orf6 protein, from *Streptomyces coelicolor*, which showed 39% identity to TcmH and catalyzed the oxidation of 6-deoxydihydrokalafungin to dihydrokalafungin [382,388–390]. This enzyme was suggested to have a relaxed substrate specificity, since in addition to its tetracyclic natural substrate, it also oxidizes tricyclic anthrones [389]. The ActVA-Orf6 protein is a homodimer with a ferredoxin-like fold and, contrary to TcmH—which possess catalytically relevant sulfhydryl groups—it does not contain any cysteine residue. Other homologous monooxygenases have been identified in polyketide synthase gene clusters, such as ElmH from *Streptomyces olivaceus* and anthrone monooxygenase AknX from *Streptomyces galilaeus* [391,392]. However, the structure of the quinol oxidizing protein YgiN of *E. coli*, which is similar to that of ActVA-Orf6, suggests that this family of monooxygenases is not restricted to the polyketide biosynthetic pathways of Gram-positive bacteria [393]. This type of monooxygenases may be useful biocatalysts for the modification of polyketide structures [382]. Combination of genetic modules for the synthesis of the "core polyketide" with genes coding for tailoring enzymes that catalyze hydroxylations, methylations, or glycosylations can expand molecular diversity among polyketide antibiotics and other aromatic polyketide-based pharmaceuticals [382,394–396].

1.3.2.2 Dioxygenases Dioxygenases belong to the large family of mononuclear nonheme iron-containing oxygenases, and are a heterogeneous group of enzymes that introduce two oxygen atoms into a C–C double bond. This class of enzymes contains either a high-spin ferrous site involved in O_2 activation, or a high-spin ferric site that activates substrates [144]. The former group includes extradiol dioxygenases and Rieske-type enzymes, whereas the latter comprises LOX and intradiol dioxygenases [208]. The ferric site is coordinated by a variable histidine-rich ligand environment and catalyzes intradiol aromatic ring cleavage and lipoxygenation. In contrast, the ferrous site is invariably coordinated by two histidines and one aspartate or glutamate, which is a recurring motif referred to as the 2-His-1-carboxylate facial triad [144]. The remaining three ligand sites, which in the resting enzyme can be vacant or occupied by water, OH^-, or protein ligands, are available for the binding of substrates, cofactors, and/or O_2 during catalysis.

Iron(III) Dioxygenases: Enzymes that Function by Substrate Activation

LIPOXYGENASE Lipoxygenase (LOX, EC 1.13.11.-) belongs to a group of enzymes found mainly in animals and plants, involved in the regulation of polyunsaturated fatty acid metabolism [397]. These enzymes are nonheme iron dioxygenases that catalyze the regio- and stereospecific hydroperoxidation of polyunsaturated fatty acids by incorporation of molecular oxygen into the 1,4-*cis*,*cis*-diene moiety of fatty acid substrates [398]. Plant LOXs usually act on linoleic acids involved in growth regulation and wound repair, whereas human LOXs catalyze the peroxidation of arachidonic acid in the biosynthesis of leukotrienes and lipoxins [399]. X-ray

structures of several soybean LOXs in the iron(II) state reveal a high-spin metal(II) center with distorted octahedral geometry [400–403]. The catalytic domain in LOXs is bigger than the *N*-terminal domain, composed roughly of seven hundred amino acids, organized in twenty-three α-helices and two small antiparallel β-sheets. Their structure reveals a nonheme mononuclear iron site within the helical bundle of the catalytic domain. The iron atom coordinates to five amino acid ligands: the imidazole *N*-atoms of three histidine ligands (His499, His504, and His690 numbering of the soybean LOX), a carboxylate oxygen of the *C*-terminal Ile839 residue, and a carbonyl oxygen of the amide backbone of a weakly bound (3 Å) Asn residue, in addition to a water molecule [397,398]. Rabbit LOX differs from the soybean enzymes in that the asparagine residue is replaced by a fourth, more strongly bound histidine residue, and it is suggested that other mammalian enzymes have similar active sites [398]. The imidazole-rich active sites give rise to a high Fe^{III}/Fe^{II} redox potential, which is proposed to be crucial for the catalytic role of the iron center in the enzyme. The iron(III) form is the catalytically active state [404,405]. The iron(II) form is activated solely by oxidation with the fatty acid hydroperoxide product [406]. The reaction profile of the iron(II)-enzyme and substrate in the presence of air shows an initial lag phase with no activity. This phase is followed by an increase in enzymatic activity as the fatty acid hydroperoxide formed by nonenzymatic auto-oxidation of the substrate, oxidizing the iron(II)-form to active iron(III)-enzyme [398]. Active LOX has a high-spin six-coordinated iron(III) metal center which is axial in the soybean enzyme, but rhombic in the rabbit LOX [406,407]. This difference has been attributed to the substitution of the weak Asn amide ligand in the former with a much stronger His ligand in the latter.

INTRADIOL-CLEAVING CATECHOL DIOXYGENASES Intradiol-cleaving catechol dioxygenases are enzymes found in an ample range of soil bacteria and are responsible for the last step in the biodegradation of aromatic rings, yielding open-chain intermediates [398]. Thus, these enzymes convert dihydroxybenzenes into acyclic compounds, which are then utilized as carbon sources for cell growth [208,398,408]. Catechol dioxygenases catalyze the oxidative cleavage of an aromatic double bond in a catechol molecule, inserting both oxygen atoms from an O_2 molecule into the product. Depending on the position of the cleaved double bond relative to the hydroxyl groups, catechol dioxygenases can be classified in two families: (i) the intradiol-cleaving catechol dioxygenases, which cleave the carbon–carbon bond of the enediol moiety (leading to muconic acids), and (ii) the extradiol-cleaving catechol dioxygenases, which cleave the bond adjacent to the enediol, yielding muconic semialdehyde adducts (Figure 1.15).

These enzymes share similar substrates, but the intradiol- and extradiol-cleaving enzymes exhibit almost complete exclusivity in their oxidative cleavage products, suggesting two different catalytic mechanisms [398]. The intradiol-cleaving catechol dioxygenases use an [Fe(III)-$(His)_2(Tyr)_2$] active site, whereas extradiol-cleaving enzymes contain a [M(II)-$(His)_2$(Asp/Glu)] motif in the active site, known as 2-His-1-carboxylate facial triad, the metal being typically iron(II), although manganese(II) is found in a few cases [408–414]. The most thoroughly studied enzyme of the class

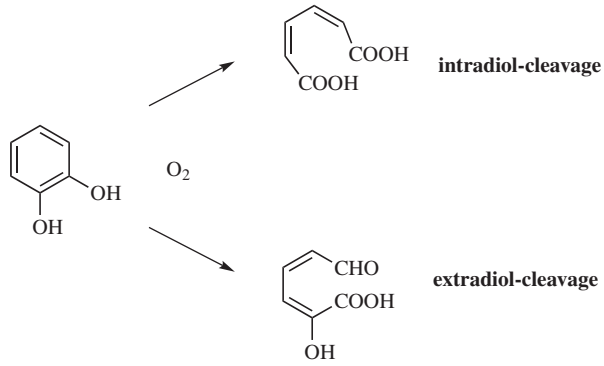

FIGURE 1.15 Intradiol vs. extradiol cleavage reaction mediated by catechol dioxygenase.

of intradiol-cleaving dioxygenases is protocatechuate (3,4-dihydroxybenzoate) 3,4-dioxygenase (3,4-PCD, EC 1.13.11.3) [398]. A number of crystallographic studies of 3,4-PCD from *P. putida* have been carried out, giving structural information of the iron(II)-state, enzyme–substrate complexes and complexes with inhibitors [415–419]. Moreover, crystal structures of the related catechol 1,2-dioxygenase from *Acinetobacter* sp. ADP1 have been solved [420]. Iron(II) 3,4-PCD from *P. putida* shows a trigonal bipyramidal iron center with four endogenous protein ligands (His460, His462, Tyr408, and Tyr447) [415,416]. The fifth coordination position located in the trigonal plane, is occupied by a solvent-derived ligand, identified as an hydroxide, thus giving rise to a charge-neutral iron(III) active site [421]. The structure of the enzyme–substrate complex reveals that the substrate binding to the iron center results in the displacement of the hydroxide and the axial Tyr447 residue [417,418]. The catechol molecule donates its protons to the displaced ligands and chelates to the metal center as a dianionic ligand. The two Fe–O bonds are *trans*- to the His460 and Tyr408 residues, and differ by 0.2 Å in length, presumably due to the distinct effects of the histidine and tyrosinate groups. The proposed catalytic mechanism postulates that the metal center retains its iron(III) character throughout the catalytic cycle (Figure 1.16) [422–424].

The first step in the catalytic mechanism is the binding of the catecholate substrate to the iron(III) center, displacing the hydroxide and the axial Tyr447 residue, and generating a square pyramidal [$Fe(His)_2(Tyr)_2$-catecholate] complex (**A**). The covalent character of the interaction iron(III)-catecholate introduces a semiquinonate radical character to the bound substrate and makes it susceptible to O_2 attack, leading to a alkylperoxo-iron(III) intermediate (**C**) *via* the transient species **B**. This intermediate then undergoes a Criegee-type rearrangement (with acyl migration) to form muconic anhydride **D** and an Fe^{III}-OH species which acts as the nucleophile to convert the anhydride into the ring-opened product **E**. Recent calculations suggest that the tridentate alkylperoxo intermediate **C** may evolve to a hydroperoxide bound in a bidentate fashion to the iron(III) center, which undergoes Criegee rearrangement to

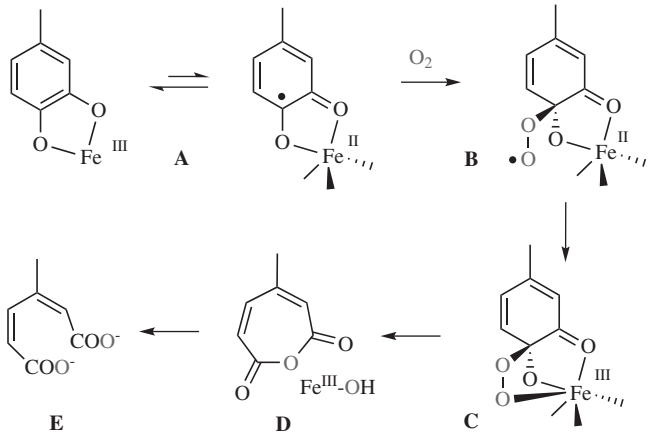

FIGURE 1.16 Proposed catalytic mechanism for intradiol-cleaving catechol dioxygenases.

yield anhydride **D** or, alternatively, the anhydride results from homolytic O–O bond cleavage and further electron transfer [425,426].

Iron(II) Dioxygenases: Enzymes with the 2-His-1-carboxylate Facial Triad Motif. Oxygen Activation The iron(II) center in these enzymes is invariably coordinated by three protein residues—two His and one Asp or Glu—arranged in one face of an octahedron, a recurring motif referred to as the 2-His-1-carboxylate facial triad [233,427]. This type of dioxygenases comprise enzymes for the biodegradation of aromatic molecules catalyzing oxidative ring cleavage, or the *cis*-dihydroxylation of arenes, as well as the superfamily of enzymes that require α-ketoacids as cosubstrates. Sequence comparisons show that the 2-His-1-carboxylate triad is conserved within each group but the sequence motif differs from each other [398]. This facial triad serves as an adequate platform for binding divalent metals. The three remaining sites on the opposite face of the octahedron are available for the binding of exogenous ligands. In the resting enzymes, these sites are usually occupied by solvent molecules but can accommodate both, substrate (or cosubstrate) and O_2 in later steps of the catalytic cycle. A general catalytic mechanism at the iron(II) center in various enzymes belonging to this superfamily has been proposed on the basis of spectroscopic and crystallographic studies [208,427,428]. The iron(II) center is six coordinated at the start of the catalytic cycle, and relatively unreactive toward O_2. Oxygen binding then initiates the oxidative mechanism, which is specific for each subclass. The metal center becomes able to bind O_2 only when substrate and cofactors are present in the active site, promoting strong coupling between the reduction of O_2 and the oxidation of substrate.

EXTRADIOL-CLEAVING CATECHOL DIOXYGENASES A second class of catechol dioxygenases includes the enzymes responsible for the extradiol cleavage of dihydroxyarenes, constituting the more common pathway for the biodegradation of aromatic molecules in soil. While the intradiol-cleaving enzymes utilize an iron(III) active

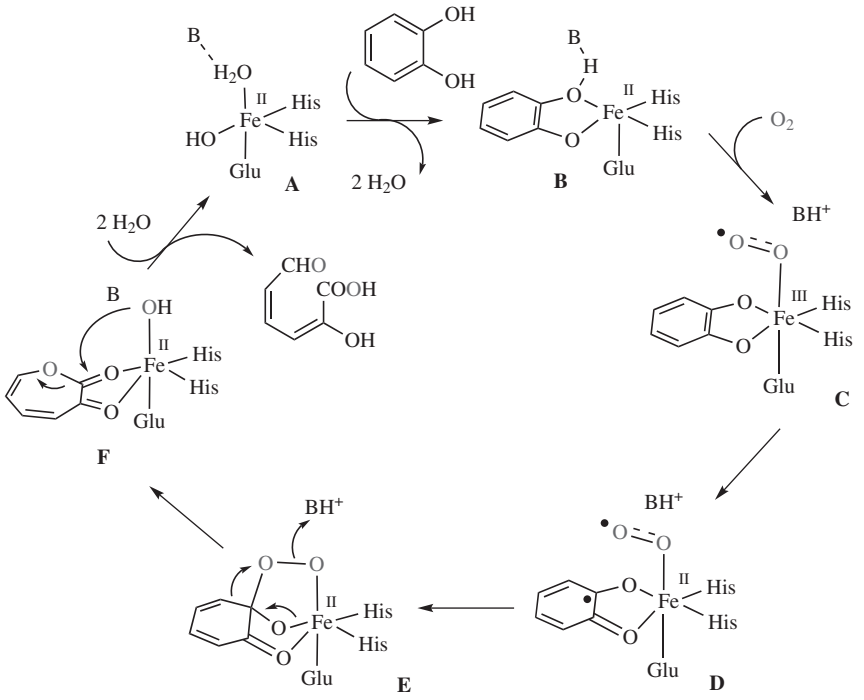

FIGURE 1.17 Proposed mechanism for the extradiol-cleaving catechol dioxygenases.

site, extradiol-cleaving dioxygenases use mainly iron(II) or else Mn(II). Crystallographic data show that the metal center is in a square pyramidal active site with a 2-His-1-carboxylate facial triad [398]. Steady-state kinetic studies revealed that this type of enzymes have an ordered catalytic mechanism with substrate binding prior to O_2 activation [409]. The coordination of the substrate serves as a trigger and significantly increases the affinity of the metal center for oxygen [429]. Crystallographic studies on 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC, EC 1.13.11.39), 3,4-dihydroxyphenylacetate 2,3-dioxygenase (HPCD, EC 1.13.11.15), and protocatechuate 4,5-dioxygenase (LigAB, EC 1.13.11.8) demonstrate that the first step of the accepted mechanism is catechol binding, resulting in the displacement of the two water ligands of the enzyme in its resting state (**A**) to form a square pyramidal metal center (**B**) (Figure 1.17) [430–434]. In the BphC structure (isolated from *Pseudomonas* sp.), one oxygen atom of the bidentate catecholate occupies the vacant position in the iron(II)-state of the enzyme, while the other occupies the site *trans*- to His210 residue, leaving the site *trans*- to Glu260 vacant [398]. The catechol binds in an asymmetric fashion to the iron(II) center as a monoion, with Fe–O bonds differing by 0.2–0.4 Å [435–438]. This monoanionic nature of the catechol substrate in extradiol dioxygenases contrast with the dianionic catecholate character commonly found in iron(III)complexes [408,409]. Oxygen binding is thus expected to occur at the vacant site *trans*- to Glu260, leading to a six-coordinated species. The change

in the covalency of the interaction (substrate-Fe vs. H_2O–Fe) would decrease the redox potential of the metal center, making it more susceptible to react with O_2. The ternary enzyme–substrate–O_2 complex thus formed (**C**) consists of the 2-His-1-carboxylate triad on one face of the metal octahedron and, on the opposite face, a bidentate monoanionic catecholate and O_2. This arrangement juxtaposes the two substrate molecules into the proper orientation for reaction. It remains unclear how the electrons flow from substrate to oxygen [398,426,439].

In one of the proposed mechanisms [398], oxygen binding to the iron(II) center results in one-electron transfer from the metal to O_2, forming the iron(III)-superoxide complex (**C**), and another one-electron transfer from substrate to metal forms a semiquinonate-iron(II)-superoxide species (**D**). The conserved His residue in the second coordination sphere may serve as the base depicted in the proposed mechanism. This residue is supposed to act later as an acid to facilitate O–O bond lysis in the ring cleavage step, and to protonate the oxide. Such oxide becomes the hydroxide ligand needed to hydrolyze the lactone. The next step in the mechanism is the nucleophilic attack of the incipient superoxide on the aromatic ring that generates an alkylperoxo intermediate (**E**), which undergoes a Criegee rearrangement to generate the seven-membered α-oxolactone ring (**F**), which hydrolyzes affording the product [398]. The alkylperoxo intermediate was observed in the crystallization of homoprotocatechuate 2,3-dioxygenase (HPCD, from *Brevibacterium fuscum*) in the presence of the low-rate substrate 4-nitrocatechol [440]. The regiospecificity of the cleavage has been rationalized invoking stereoelectronic factors (the peroxo group is positioned axially with respect to the cyclohexadiene ring providing optimum alignment for the required 1,2-shift), or acid–base catalysis [426]. However, considering the similarities to the intermediates of the intradiol-cleaving enzymes, the found regiospecificity is not fully understood, and other mechanisms have been postulated, including the formation and cleavage of an epoxide before lactone generation [398].

ENZYMES REQUIRING α-KETOACIDS AS COSUBSTRATE Enzymes requiring α-ketoacids as cosubstrates constitute the largest and most diverse family of mononuclear nonheme iron enzymes, catalyzing many pivotal metabolic transformations [441–443]. Substrate oxidation—typically involving (but not limited to) the functionalization of an inactivated C–H bond—occurs with the oxidative decarboxylation of an α-ketocarboxylate, such as 2-oxoglutarate. For the hydroxylation, one atom of dioxygen is incorporated into the product, while the other atom ends up on the carboxylate derived from the ketoacid, constituting an intermolecular variant of the dioxygenase reaction (Figure 1.18) [398].

Moreover, in other oxidative processes catalyzed by these enzymes, only one of the dioxygen atoms is finally incorporated into the secondary substrate (yielding succinate), while the other one is reduced to water. As an example, the primary

FIGURE 1.18 Hydroxylation of unactivated C–H bonds mediated by α-ketoacid-dependent dioxygenases.

substrate undergoes a two-electron oxidation without oxygen incorporation to form a ring (clavaminate synthase, which catalyzes the three steps: (i) hydroxylation, (ii) cyclization, and (iii) desaturation), or a ring expansion during antibiotic biosynthesis (deacetoxycephalosporin C synthase) (Figures 1.19A and B, respectively).

α-Ketoacid-dependent enzymes are found in microorganisms, plant and animals, and play important roles in biochemical pathways that have environmental, pharmaceutical, and biological significance [398]. Enzymes of this family also appear in microbes that use alternative energy sources, such as sulfonates and hypophosphites. For instance, taurine/2-OG dioxygenase (TauD, EC 1.14.11.17) enables *E. coli* to use the aliphatic sulfonate taurine as a sulfur source during periods of sulfate starvation [444]. Despite the vast different transformations these enzymes are able to carry out, the members of this family require one equivalent of iron(II), an α-ketoacid, dioxygen, and ascorbate for full activity. Crystal structures of a number of different 2-oxoglutarate-dependent enzymes show a double-stranded β-helix as common architecture [445–452]. While many of these enzymes show minimal sequence identity, there is a high degree of sequence homology. The conserved residues of the facial triad bind the high-spin iron(II) center, while other conserved arginine, lysine, and serine residues serve to bind the anionic C-5 carboxylate of 2-oxoglutarate [398]. However, several enzymes in this class have substrates with a built-in α-ketoacid function, and thus do not require 2-oxoglutarate as cosubstrate. These enzymes appear to have different topologies from enzymes utilizing 2-oxoglutarate, and

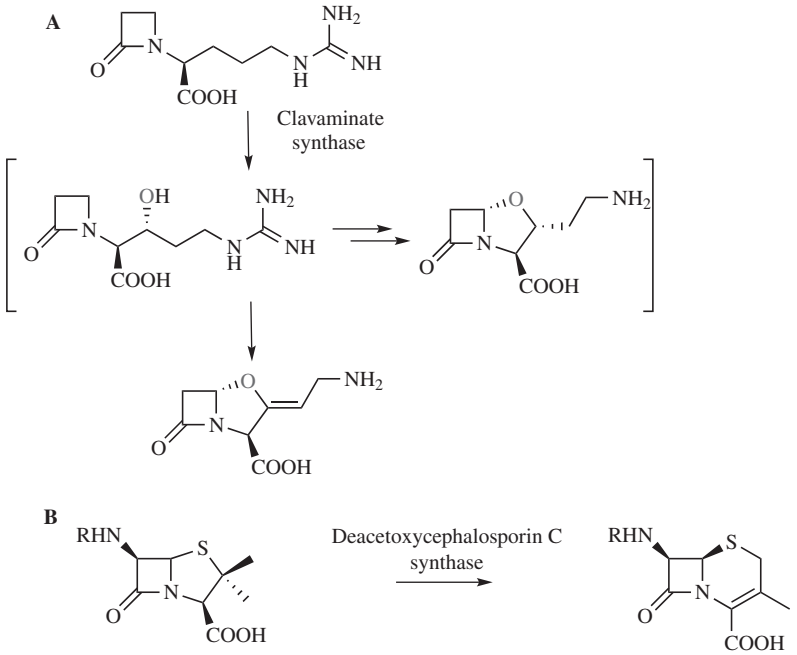

FIGURE 1.19 Two-electron oxidations mediated by α-ketoacid dependent dioxygenases. **A**: Ring formation without oxygen incorporation. **B**: Ring expansion.

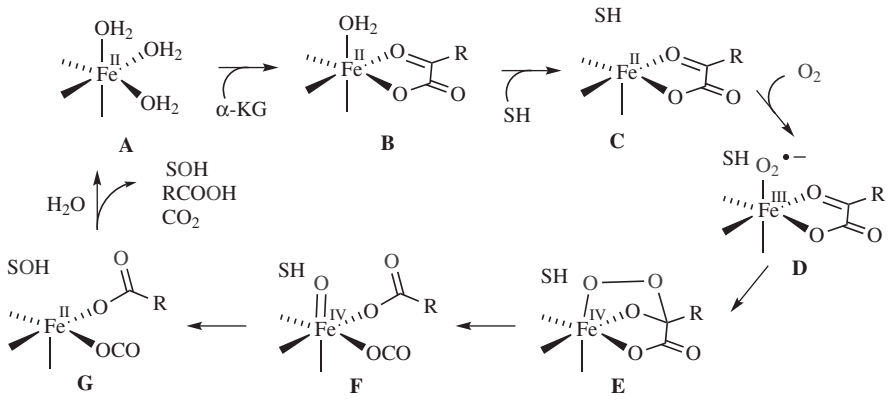

FIGURE 1.20 Proposed reaction mechanism for the α-ketoacid-dependent enzymes.

include 4-hydroxyphenylpyruvate dioxygenase (HPP dioxygenase, EC 1.13.11.27) and 4-hydroxymandelate synthase (EC 1.13.11.46), which utilize the same substrate but catalyze different transformations. A common mechanism was proposed for α-ketoacid-dependent iron oxygenases (Figure 1.20) [208,408,443], in which the iron(II) center in the resting enzyme is ligated to the 2-His-1-carboxylate facial triad and three water molecules complete the coordination sphere (**A**) [453–455].

The α-ketocarboxylate binds orderly, prior to binding of either dioxygen or substrate, by displacement of two water molecules, leading to a six-coordinated iron(II) center with a bidentate α-carboxylate (**B**) [445,446,449,452,456–462]. The equatorial plane of the distorted octahedron is composed of the nearly planar five-membered ring of the chelated α-ketocarboxylate, as well as a histidine and a carboxylate of the facial triad, with the α-keto- group bound in *trans* conformation to the aspartate or glutamate residue. Apical sites are occupied by the remaining histidine residue and one water molecule, which appear to have hydrogen-bonding interaction with the carboxylate residue. The next step in the catalytic cycle is still under discussion. Oxygen binding may occur prior to substrate binding, or the sequence may be the opposite, with substrate binding occurring prior to oxygen [456,457,462–466]. Latter studies show that substrate binding results in the formation of a coordinatively unsaturated iron(II) center, presumably by loss of the remaining water ligand, that primes the metal center for oxygen binding and activation [446,448], supporting the proposed substrate-induced conversion of a six-coordinated to a five-coordinated iron center. Substrate binding may induce a shift of the carboxylate residue, removing the hydrogen-bonding interaction with the water ligand in the binary complex, thus facilitating its withdrawal. In this mechanistic proposal, the binding of dioxygen leads to an adduct with significant iron(III) superoxide radical anion character [456,467,468]. The oxidative decarboxylation of the α-ketoacid can be uncoupled from substrate oxidation, particularly when no substrate or a poor substrate analog is employed [456,469–471]. The attack of O_2 on the coordinated α-ketoacid leads to the formation of a high-valent intermediate responsible for the oxidation of the substrate in α-ketocarboxylate-dependent enzymes. Such species has been postulated as an

iron(IV)-peroxo species (**E**) that can lose CO_2 to form an iron(II)-peracid adduct and then undergo heterolytic cleavage of the O–O bond to yield an iron(IV)-oxo species (**F**). Substrate hydroxylation is postulated to occur *via* an analogous mechanism to that of cytochrome P450, that is, a two-step process involving hydrogen atom abstraction by the oxo-iron(IV) species followed by oxygen rebound [398]. The difference is that P450 uses a formal Fe(III)/Fe(V)=O couple, while the α-ketoacid-dependent enzymes must use a formally Fe(II)/Fe(IV)=O couple. Carbon dioxide is a product of reactions catalyzed by these classes of enzymes, and it was suggested that CO_2 dissociation follows release of the hydroxylated product [457,459]. For enzymes catalyzing ring cyclization or desaturation instead of hydroxylation, the rebound step is replaced by a step which involves either oxidative ligand transfer to close a ring, or a second hydrogen-atom abstraction to form a double bond [398].

RIESKE-TYPE DIOXYGENASES The hydroxylation of aromatic rings is the most difficult catalytic step in the aerobic degradation of aromatic compounds [472]. Bacterial Rieske-type nonheme dioxygenases catalyze the stereoselective introduction of two hydroxyl groups into the aromatic ring. Rieske-type dioxygenases (NAD(P)H dependent) play important roles in the biosynthesis of secondary metabolites such as flavonoids and alkaloids, and in performing a key step in the natural degradation of aromatic compounds [473–475]. These enzymes display broad substrate specificity catalyzing highly enantiospecific hydroxylations. These features make them attractive tools for the preparation of synthons for the production of industrially useful chiral chemicals [145,476]. Microbial dioxygenases catalyze a key step in the degradation of aromatic compounds pathway, which is the *cis*-dihydroxylation of arenes. Wild-type strains of microorganisms rapidly metabolize *cis*-diols into aromatic compounds, *via* a dihydrodiol dehydrogenase-mediated reaction, with the subsequent loss of chirality. However, in Gibson's blocked mutant *P. putida* 39/D, the expression of dihydrodiol dehydrogenase is knocked out so that the intermediate *cis*-dihydrodiol accumulates in the culture broth [477]. The use of mutant strains with suppressed dehydrogenase activity, allows the high yielding isolation for further use in synthesis of the accumulated *cis*-dihydrodiols [473,478,479]. The most important enzymes in this group are arene dioxygenases (EC 1.14.12.-), which catalyze the conversion of aromatic compounds into vicinal *cis*-diols, and are produced exclusively in bacteria (*Pseudomonas*, *Rhodococcus*, *Sphingomonas*). Their broad substrate specificity enables them to catalyze enantiospecific reactions on a wide variety of aromatic compounds such as benzene, naphthalene, biphenyl derivatives, and phthalic and benzoic acids [478,479]. Over 100 arene dioxygenases have been identified so far [480]. These Rieske nonheme iron oxygenases (ROs) comprise actually two/three components: (i) a reductase domain which obtains electrons from NAD(P)H; (ii) often a Rieske ferredoxin component that shuttles the electrons; (iii) and an oxygenase component that performs catalysis. The oxygenase component structures have all shown to be of the $\alpha 3$ or $\alpha 3\beta 3$ types, containing a mononuclear iron site and a Rieske-type iron–sulfur cluster [2Fe–2S] in each α subunit. The transfer of electrons takes place from the Rieske center to the mononuclear iron of the neighboring subunit *via* a conserved aspartate residue, which is shown to be involved in gating electron transport [208,476,481].

Dioxygenases were initially classified on the basis of the electron transfer components present in the oxygenase system. Two component dioxygenases (reductase and oxygenase) were classified as Class I, and three component enzymes (reductase, ferredoxin and oxygenase) were classified as Class II and III. This classes were further subdivided based on the type of flavin cofactor (FAD or FMN) in the reductase, the presence or absence of an iron–sulfur center in the reductase, the number of proteins in the oxygenase, and if a ferredoxin was involved, the type of iron–sulfur center in the ferredoxin [472]. A second classification system, based on the phylogeny of the oxygenase α subunit was proposed later, and four families were distinguished: Toluene/biphenyl, naphthalene, benzoate, and phthalate dioxygenases [472,476,482]. This classification system was based on the catalytic activity of the enzymes, because α subunit of the oxygenase plays an important role in determining substrate specificity. A new family has emerged recently, consisting of enzymes that catalyze the oxidation at either the 1- or 5-position of salicylate as well as other substrates, and was designated as the salicylate family [483–486]. However, there are still members that do not belong to any of these families, such as enzymes that oxygenate aniline, dibenzodioxins, 3-phenylpropionate, or *o*-benzoate.

Some Gram-positive bacteria such as *Rhodococcus opacus*, are also capable to use polycyclic aromatic hydrocarbons (dibenzofurans, dibenzo-*p*-dioxins) as carbon sources, by producing a unique arene dioxygenase which catalyzes side dioxygenation reactions [487]. Consequently, a new classification was recently put forward [488]. In addition, arene dioxygenases have been classified in many types (namely toluene-, chlorobenzene-, phthalate-dioxygenase, etc.) based on the arene used as a carbon source. The most widely used types in biotransformations include benzene- (BDO), toluene- (TDO), biphenyl- (BPDO), chlorobenzene- (CDO), benzoic acid- (BZDO), nitrobenzene- (NBDO), and naphthalene-dioxygenase (NDO). Because of the multicomponent nature of these ROs and the need of cofactors, the biotransformations are performed using whole-cell biocatalytic systems. The overall reaction stoichiometry for the arene dioxygenation (Figure 1.21) requires molecular dioxygen and two electrons that come from a NAD(P)H reductase and are transferred to the final oxygenase component through a ferredoxin (in the two-component dioxygenase systems, the ferredoxin is absent).

A large amount of structural information is now available in the Protein Data Bank for several members of this family of ROs. The most thoroughly studied dioxygenase is naphthalene 1,2-dioxygenase (NDO), which catalyzes the NAD(P)H and O_2-dependent oxidation of naphthalene to (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene and is used as a model and as a basis for comparison to other related enzyme systems [489–496]. NDO consists of three protein components: (i) a flavo-[2Fe–2S] reductase [497], (ii) a Rieske ferredoxin electron transfer protein

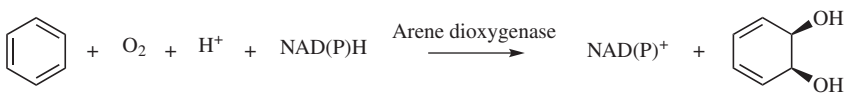

FIGURE 1.21 Stoichiometry of the arene dioxygenation.

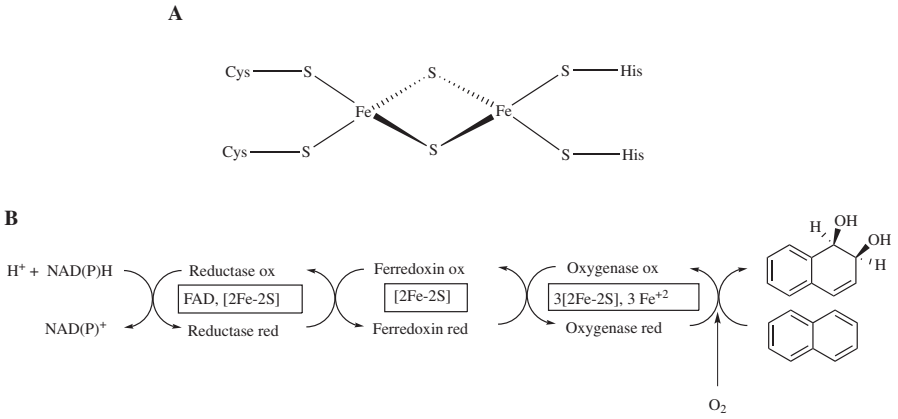

FIGURE 1.22 **A**: Rieske-type [2Fe–2S] cluster in the active site of NDO. **B**: Electron transfer in *P. putida* NDO-mediated dihydroxylation of naphthalene.

[498], and (iii) an $\alpha 3\beta 3$ oxygenase containing a mononuclear iron(II) center and a Rieske-type [2Fe–2S] cluster in each α-subunit (Figure 1.22A). The active site is built across a subunit–subunit boundary, and each subunit contributes with one type of metal center (Figure 1.22B) [481,495,496,499,500].

The rational basis on how the electrons are transferred from the reductase to the ferredoxin and from this to the oxygenase has advanced substantially with the disclosure of structures of biphenyl- and carbazole-dioxygenases [426,501]. Conformational changes in the flavin ring give rise to a direct electron transfer to the Rieske cluster of the ferredoxin [496]. From studies on carbazole dioxygenase, it is likely that there is a gating mechanism that senses the conformational state of the iron cofactor to regulate the transfer of the electron from the ferredoxin to the Rieske cluster of the neighboring subunit [426]. The active site of NDO from *Pseudomonas* sp. NCIB 9816-4 contains a hydrophobic pocket with a mononuclear iron(II) center, coordinated by His208, His213, and a bidentate Asp362 (the 2-His-1-carboxylate facial triad [233], present in all ROs) [502]. The mononuclear iron(II) center is positioned 12 Å from the [2Fe–2S] cluster of another subunit in the $\alpha 3\beta 3$ oxygenase domain, which delivers two electrons to the dioxygenase active site during each turnover. Structure determination of the ternary complexes with substrates (naphthalene or indole) and dioxygen have shown that dioxygen is bound side-on to the iron(III) center, an unusual binding mode for dioxygen, and that the aryl substrate is positioned 4.3 Å from the iron center, beyond and parallel to the bound oxygen [496]. An important difference in the geometry of the active sites of cytochrome P450 and NDO is that the active-site iron is highly accessible in NDO, with a large part of its surface available for the binding of both oxygen atoms to iron. Structures of other Rieske dioxygenases have been determined recently, and are similar to that of the NDO enzyme [426,472,481]. Detailed low temperature crystallographic studies of NDO and its complexes with either oxygen or substrates or both, have provided insights into possible catalytic mechanisms [496]. The structure, function, and constitution of

the dioxygenase three-component protein, including cofactors and electron-transport chain, are relatively well understood. However, the precise catalytic mechanism for *cis*-dihydroxylation is not clear, and different theories have been developed regarding the oxidation state of iron during catalysis [481,489,490].

The catalytic cycle of arene dioxygenases may involve two steps: the activation of molecular oxygen, and the addition to the substrate. Some studies suggested that the activation of oxygen in the active site of the oxygenase goes through Fe^{4+} and Fe^{5+}. On the other hand, this activation was suggested to take place through an Fe^{3+}–hydroperoxide complex [R–Fe^{3+}–OOH], and currently this is the accepted mechanism [481,503,504]. The involvement of [2Fe–2S] iron–sulfur clusters implies that single electron transfer events take place in the catalytic cycle. Single turnover studies of the NDO reaction have shown that turnover requires reduced enzyme and bound substrate, and that the mononuclear iron(II) center and one Rieske [2Fe–2S] cluster are oxidized during turnover, resulting in iron(III) at the end of the catalytic cycle [489]. The original crystal structure solved for NDO contained an indole hydroperoxide ligand, ligated to the iron center, with the indole ring positioned at about 4 Å from the iron center [504], possibly indicating the existence of a hydroperoxide intermediate in the catalytic mechanism. Hydrogen peroxide is released by NDO if benzene is used as substrate, suggesting that dioxygen is activated *via* reduction to superoxide, which in this case is uncoupled from substrate hydroxylation and is further reduced to peroxide [505]. Recent studies on benzoate 1,2-dioxygenase have shown that enzyme containing a fully oxidized Rieske cluster can use hydrogen peroxide to form *cis*-diol products [506]. In the same way, the observation of monooxygenase activity with certain substrates, suggests that arene *cis*-dihydroxylation is not a concerted process, and that high-valent iron-oxo intermediates may be involved in monohydroxylation [507]. Despite these different mechanistic interpretations [508,509], each of these observations is consistent with an iron(III) hydroperoxy intermediate (Fe^{3+}–OOH), which might either be the active oxidant, reacting directly with the aryl substrate, or undergo O–O bond homolysis to form a reactive O=Fe(V)–OH species [489] (Figure 1.23) [426]. The oxidant could theoretically react with the aryl substrate *via* a cationic or a radical mechanism. Recent radical trap studies showed that the monooxygenation of norcarane using NDO occurs *via* radical intermediates, remaining unclear whether the dioxygenation reaction also proceeds in the same way [510]. It seems that the gathered evidence points to a radical mechanism, but clearly further experimental and theoretical studies are needed to unravel the precise mechanism of the dihydroxylation of arene substrates.

NDO is a relatively specific enzyme, capable to hydroxylated benzene, toluene, and substituted phenols, incorporating the oxygen atoms not only into the *o*-position, but also into the *p*-position [491]. Furthermore, NDO can catalyze monohydroxylations, sulfoxidation, desaturation reactions (formation of C–C double bonds), dehydrogenations, and *O*- and *N*-dealkylations, in a similar way as CYP450 (see Chapter 4) [492,493]. Due to their great versatility, arene dioxygenases are biocatalysts with enormous potential in biotechnological applications, but considering their complexity and cofactor NAD(P)H requirements, their utilization is highly focalized toward the whole-cell biotransformations [37].

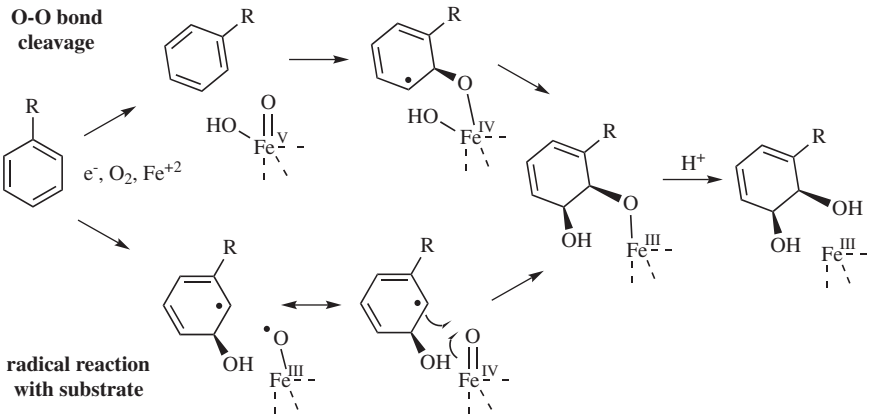

FIGURE 1.23 Proposed radical-based dioxygenase-mediated mechanism for *cis*-hydroxylation of arenes [426].

1.3.3 Oxidases

Oxidases (EC 1.1.3.-, 1.2.3.-, 1.3.3.-, 1.5.3.-, 1.10.-, 1.13.-, and 1.14.-) catalyze oxidation reactions using molecular oxygen as the electron acceptor and reducing it either to hydrogen peroxide or to water, without the incorporation of oxygen atoms into the substrate [149,199]. These enzymes do not require cofactors for developing their catalytic cycle, and use oxygen as electron acceptor in the oxidation of alcohols. This no need of cofactors makes the use of oxidases in biocatalytic processes more straightforward than the use of cofactor dependent enzymes such as dehydrogenases or oxygenases. Most oxidases are not involved in biosynthetic reactions, but play a key role in metabolism serving as terminal electron acceptors in energy storage pathways [144]. However, there are oxidases that make use of the oxidizing potential of O_2 to promote biosynthetic reactions. These enzymes may couple one-, two-, or four-electron oxidation of substrates to the two- or four-electron reduction of O_2 to H_2O_2 or H_2O, not requiring the external addition or regeneration of coenzymes. Oxidases catalyze a wide variety of synthetically interesting oxidations including the oxidation or amines, the oxidation of alcohols including sugars, oxidative ring closures, or oxidative decarboxylations, and can be divided into iron- or copper-containing oxidases, and flavin-dependent enzymes.

1.3.3.1 Iron-Containing Oxidases This type of oxidases belong to the mononuclear nonheme iron enzyme family, featuring the 2-His-1-carboxylate facial triad [439,511]. Mechanisms of oxygen activation proposed for this subgroup of enzymes are diverse, but the general strategy appears to involve the binding of both the substrate and O_2 to the iron, similar to extradiol-cleaving and 2-oxo acid dioxygenases, which belong to the same family of enzymes [144]. This promotes the electron transfer away from the substrate and, in most cases, the iron. The reduced dioxygen species undergoes O–O bond cleavage yielding a water molecule

and leaving an Fe(IV)=O species as in 2-oxo acid-dependent oxygenases and pterin-dependent hydroxylases. Finally, the high-valent iron species is used as a reagent completing the second part of the reaction, and accepting two more electrons to form the second molecule of water. Examples of oxidases featuring the 2-His-1-carboxylate facial triad are isopenicillin *N*-synthase (IPNS, EC 1.21.3.1) [512,513], (*S*)-2-hydroxypropylphosphonic acid epoxidase (HppE, EC 1.14.19.7) [514], and ethylene forming enzyme 1-aminocyclopropane-1-carboxylate oxidase (ACCO, EC 1.14.17.4) [515]. These enzymes activate O_2 and ultimately form water using either four electrons from the substrate (IPNS), two from the substrate and two from NADH (HppE), or two from the substrate and two from ascorbate (ACCO).

1.3.3.2 Copper-Containing Oxidases These enzymes catalyze the oxidation of a wide variety of substrates ranging from small molecules (e.g., methane) to large peptides, with concomitant reduction of oxygen to hydrogen peroxide or water. The copper sites of these enzymes have been historically divided into three classes based on their spectroscopic features (reflecting the geometric and electronic structure of the active site): (i) type 1 (T_1) or blue copper; (ii) type 2 (T_2) or normal copper; and (iii) type 3 (T_3) or coupled binuclear copper centers [350]. Along these lines, copper-containing oxidases can be divided in four groups: (i) mononuclear enzymes containing sulfur-ligated copper (type 1); (ii) mononuclear copper oxidases with non-sulfur ligation (type 2); (iii) dinuclear copper oxidases (type 3); and (iv) multicopper oxidases [366].

Type 1 copper oxidases, actually functioning not as oxidases but as electron transfer proteins, are also called "blue-copper proteins" because of their intense color arising from a Cu(II)→S(cysteine) charge transfer transition. Amine oxidases (AOs) can be classified on the basis of their cofactor dependence, as either copper- (formerly EC 1.4.3.6, now EC 1.4.3.21 and EC 1.4.3.22) or flavin-containing (EC 1.4.3.4) enzymes [516]. These enzymes catalyze the oxidation of primary amines to aldehydes, with the subsequent release of ammonia and hydrogen peroxide. In Nature, they catalyze the oxidative deamination of a wide range of biogenic amines including many neurotransmitters, histamine, and xenobiotic amines [366,517,518].

Copper-containing amine oxidases (CuAO) are typical type 2 copper oxidases, occurring in both prokaryotes and eukaryotes. Enzymes in prokaryotes (e.g., *E. coli*) allow the organism to use various amine substrates as source of carbon and nitrogen. In higher eukaryotes their biological functions are less well defined, with suggested roles for detoxification and cell signaling in animals and cell wall cross-linking, lignifications and defense in plants [516]. The CuAO from *E. coli* is a 150–190 kDa homodimer containing two distinct active sites [518–523], each one contains a copper ion and an organic cofactor, 2,4,5-trihydroxyphenylalanine quinone (TPQ), which is derived from the posttranslational modification of an endogenous tyrosine residue [516,524]. The two-electron chemistry is carried out at a redox site consisting of a mononuclear Cu(II) ion and TPQ. The catalytic cycle initiates with the reductive half-reaction in which TPQ in the resting enzyme undergoes nucleophilic attack by the primary amine substrate, thus forming a Schiff-base intermediate (**B**) followed by an aspartate-mediated proton abstraction to generate a carbanionic intermediate (**C**)

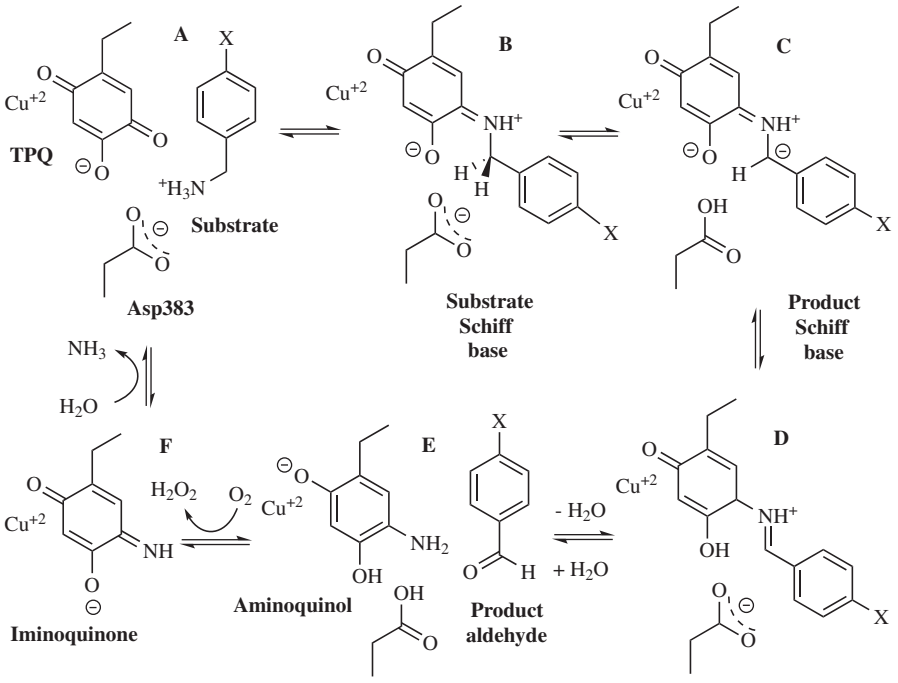

FIGURE 1.24 Catalytic mechanism for *E. coli* CuAO-catalyzed reaction.

(Figure 1.24). Further rearrangement and hydrolysis lead to the release of product aldehyde and the formation of aminoquinol (**E**). The oxidative half-reaction uses O_2 to regenerate the resting enzyme, probably *via* an iminoquinone intermediate (**F**) to release hydrogen peroxide [525]. Further hydrolysis of the mentioned intermediate releases NH_3 and regenerates the cofactor in the resting enzyme (**A**). However, the oxidative half-reaction has remained elusive in terms of oxygen activation and the relative roles of both the copper and TPQ in this process [518]. It seems clear that copper is not required to change its redox state during catalysis, and it has been suggested that its principal role is in electrostatic stabilization [526–529].

Galactose oxidase (GAO, EC 1.1.3.9) is the best characterized enzyme of the family of extracellular mono-copper oxidases of fungal origin. It was first described from *Fusarium* spp. [530–533], and later, its catalytic mechanism [534,535], structure [536,537] and substrate range [538–540], were thoroughly studied. The enzyme has a molecular weight of 68.5 kDa and is composed of 639 amino acidic residues [535]. Its physiological role, still not clear, could be involved in the production of hydrogen peroxide for further use as a co-oxidant for lignin- and cellulose-degrading peroxidases and/or as an antibiotic defense within the rhizosphere [199]. GAO catalyzes the oxidation of D-galactose at the C-6 position in the presence of oxygen to give D-galactohexodialdose and H_2O_2 as by product, which is usually destroyed by catalase to avoid inactivation of the enzyme. These enzymes perform two-electron redox chemistry, coupling the oxidation of the primary alcohol to the corresponding

aldehyde with the reduction of O_2 to H_2O_2 [541,542]. The reaction stops at the oxidation stage of the aldehyde, and does not proceed to the corresponding acid. The two-electron oxidation catalyzed by a single copper atom is explained by the presence of a tyrosyl radical unit in the protein active site, acting as a second redox center during the catalytic cycle. The crystal structure of GAO from the fungus *Dactylium dendroides* was published in 1991, and provided new directions for mechanistic studies [543]. A Cu(II) ion is equatorially ligated by His496, His581, and Tyr272 residues and a solvent molecule. An axial Tyr495 completes the distorted square pyramidal geometry. Tyr272 is cross-linked to Cys228 *via* a unique thioether bond to create the radical-forming cofactor in active GAO. In addition, the indole ring of Trp290 is stacked above the thioether bond. As isolated, the enzyme is a mixture of inactive, semireduced Cu(II)-Tyr, and active, oxidized Cu(II)-Tyr• [366]. For oxidized GAO, the visible absorption band at 810 nm arises from a copper-mediated Tyr495 to Tyr272• charge transfer transition. In the semireduced enzyme, the absorption band at 458 nm was attributed to a Tyr272 to Cu(II) ligand-to-metal charge transfer transition. The data also suggested that Trp290 acts to stabilize the radical cofactor [544]. GAO also accepts as substrates carbohydrates containing an accessible D-galactose moiety such as galactosides, melibiose, raffinose, lactose, and lactitol [199]. Furthermore, the stereospecific oxidation of glycerol, 3-halogenopropane-1,2-diols, and polyols to the corresponding aldehydes was described using the commercially available enzyme from *D. dendroides* [545,546]. On the other hand, the enzyme cannot oxidize non-D-galactose-based carbohydrates, whereas it could accept shorter polyol chains, such as glycerol, which could be twisted to form a conformation similar to the D-galactose C4–C6 fragment [547].

Type 3 (dinuclear copper-oxidases) and multicopper oxidases catalyze the four-electron reduction of O_2 to water coupled to the concomitant one-electron oxidation of the substrate [144,149]. Tyrosinases (EC 1.14.18.1) are prominent examples of type 3 copper oxidases (although they can also be classified as monooxygenases), catalyzing the oxidation of both monophenols (known as cresolase or monophenolase activity) and *o*-diphenols (catecholase or diphenolase activity) into *o*-quinones (see Section "Copper-containing monooxygenase") [368]. In the cresolase cycle, the four-electron reduction of one molecule of O_2 is coupled to the four-electron oxidation of one monophenol unit *via* diphenol to an *o*-quinone, whereas in the catecholase cycle, it is coupled to the successive two-electron oxidation of two diphenol molecules [120]. Laccases (EC 1.10.3.2) are conspicuous examples of multicopper oxidases. They act *via* a free-radical mechanism, using molecular oxygen in relatively non-specific reactions [548]. The enzyme couples the monoelectronic oxidation of four equivalents of the substrate, with the transfer of four electrons to molecular oxygen, together with its reduction to water [549]. The enzymes are extracellular glycoproteins secreted by white-rot fungi, usually occurring as isoenzymes that oligomerize to form multimeric complexes, and act on phenolic substrates catalyzing the oxidation of hydroxyl groups to phenoxyl radicals. Further nonenzymatic transformations result in lignin degradation [550]. The molecular weight of the monomer ranges from 50 to 130 kDa. The carbohydrate moiety of laccases, which is believed to contribute to the stability of the enzyme, typically consists of mannose, *N*-acetylglucosamine,

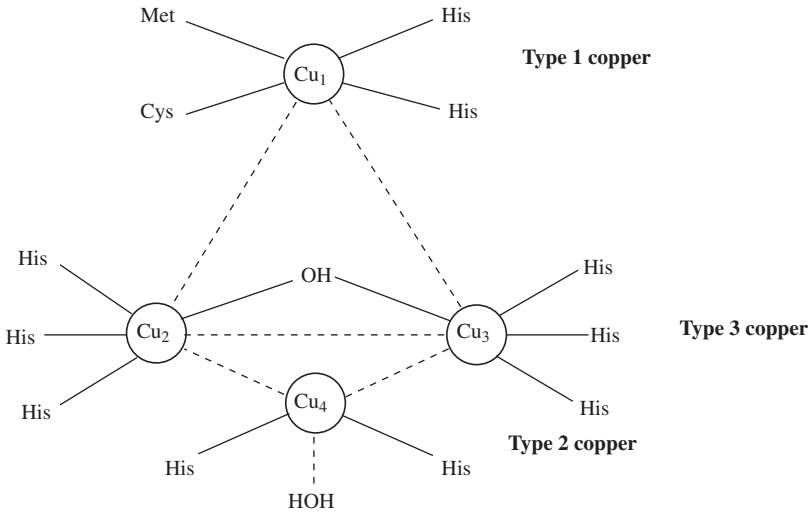

FIGURE 1.25 Schematic representation of the active site of laccase CotA from *Bacillus subtilis*.

and galactose, ranging from 10% to 45% of the protein mass [551]. The active site of laccase contains at least four copper atoms, which are one type 1 (T_1) and a trinuclear cluster (T_2/T_3) as the minimal functional unit, consisting of one type 2 (T_2) and two type 3 (T_3) copper atoms [350,552]. The T_1 copper atom is located at a distance of approximately 12 Å from the T_2/T_3 site, and the T_2 copper atom is located at 4 Å from the T_3 copper atoms (Figure 1.25).

The T_1 copper has a trigonal coordination with two histidines and one cysteine, and the axial ligand is methionine in the bacterial (CotA) enzyme and leucine or phenylalanine in fungal laccases [552]. This copper atom is the one which confers the typical blue color to multicopper proteins (also called "blue copper proteins") due to the strong adsorption around 600 nm, caused by the covalent copper-cysteine bond. Substrate oxidation takes place at this type 1 site due to two contributing factors, namely its high redox potential and the easy accessibility to the active site from the surface (compared to the limited substrate access to the trinuclear cluster). Type 2 copper coordinates with two histidines, while each T_3 atom is coordinated to three histidine ligands. Both take place at the trinuclear cluster (T_2/T_3 site) the reduction of molecular oxygen and release of water. The catalytic properties of laccase have been attributed to three major steps [350,552]: (i) type 1 copper is reduced by accepting electrons from the reducing substrate; (ii) electrons are transferred ∼13 Å from type 1 copper to the trinuclear T_2/T_3 cluster; and (iii) molecular oxygen is activated and reduced to water at the T_2/T_3 cluster. During the catalytic cycle of laccase-mediated reactions, the dioxygen molecule interacts with the completely reduced trinuclear cluster T_2/T_3 *via* a two-electron process to produce the peroxide intermediate which contains the dioxygen anion (Figure 1.26) [553]. One oxygen atom of the dioxygen anion is bound to the T_2 and T_3 copper ions and the other oxygen atom is coordinated

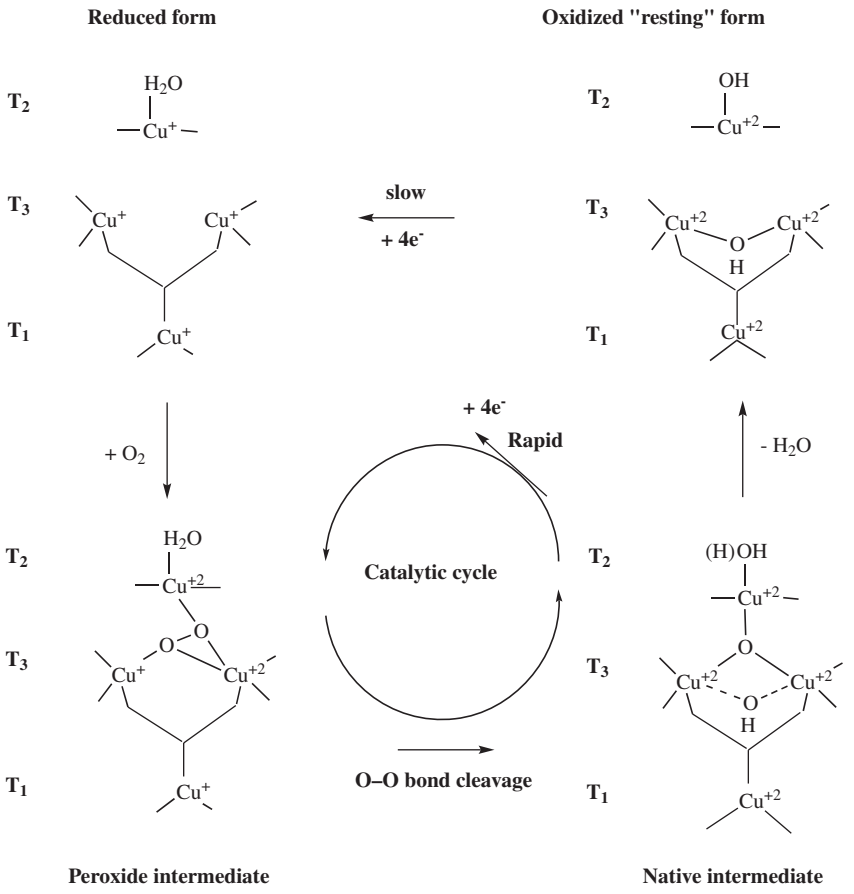

FIGURE 1.26 Catalytic cycle of laccase showing the mechanism of four-electron reduction of a dioxygen molecule to water at the enzyme copper sites.

with the other T_3 copper. Then, the peroxide intermediate undergoes a second two electron process and the peroxide O–O bond breaks to produce an intermediate, termed native intermediate, which is the fully oxidized form with the three copper centers in the trinuclear site bridged by the product of full O_2 reduction with at least one Cu–Cu distance of 3.3 Å. Solomon and coworkers confirmed the structure of this native intermediate by the combination of X-ray spectroscopy and magnetic circular dichroism [554,555].

The three copper centers in the trinuclear cluster are all bridged by a μ_3-oxo ligand at the center of the cluster, with the second oxygen atom from O_2 either remaining bound or dissociated from the trinuclear site. This structure provides relative stability, which is the thermodynamic driving force for the four-electron process for oxygen reduction and provides efficient electron transfer pathways from the T_1 site to the other copper center in the trinuclear cluster [555]. This electron transfer leads to the

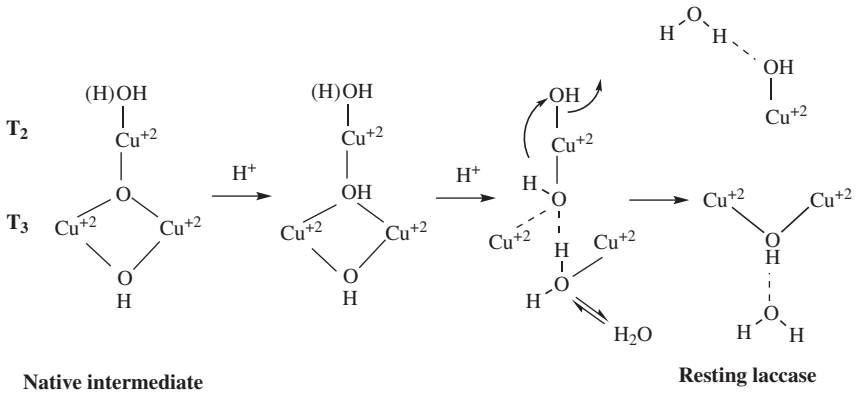

FIGURE 1.27 Proposed decay mechanism of the native intermediate to the resting laccase.

fast reduction of the complete oxidized trinuclear cluster in the native intermediate, thus generating the fully reduced site in the reduced form for further turnover with O_2. The native intermediate slowly converts to a completely oxidized form called "resting" laccase, which has the T_2 copper isolated from the coupled binuclear T_3 centers. This rearrangement only occurs in the absence of reductant and the decay of the native intermediate to the resting enzyme proceeds *via* successive proton-assisted steps (Figure 1.27) [556]. The first proton binds at the μ_3-oxo center and then the second proton binds at the T_3 OH^- bridge. Finally, the three copper centers in the trinuclear cluster are uncoupled to form the resting form of the enzyme.

Since oxidase catalysis is typically not dependent on an additional electron source such as NAD(P)H and thus it does not require the addition of an organic cosubstrate for its regeneration, these enzymes are usually applied *in vitro*, where they are independent from cell metabolism. However, enzyme deactivation by reactive oxygen species and the addition or removal of hydrogen peroxide are most critical issues that have to be considered [144].

1.3.3.3 Flavin-Dependent Oxidases Among flavin-dependent enzymes, the vanillyl alcohol oxidase (VAO, EC 1.1.3.38) family of flavoproteins containing a covalently bound FAD cofactor, constitutes a prominent and synthetically interesting group [557]. As an example, VAO from *Penicillium simplicissimum* catalyzes both the benzylic desaturation and the benzylic hydroxylation of *p*-alkylphenols, depending on the nature of the aliphatic chain [558,559]. The typical VAO-catalyzed reaction is the oxidation of vanillyl alcohol (4-(hydroxymethyl)-2-methoxyphenol) to vainillin. However, the enzyme is also able to catalyze deaminations, hydroxylations, as well as other dehydrogenation reactions, frequently giving flavor compounds. For the benzylic desaturation and hydroxylation of *p*-alkylphenols, the catalytic mechanism involves the substrate oxidation by the flavin to *p*-quinone methides as common intermediates, and differs in whether water attacks the methide. Thus, in contrast with flavin-dependent oxygenases, the flavin is not reduced by NAD(P)H but directly

by the substrate and the introduced oxygen atom, in case of benzylic hydroxylation, is derived from water as in the case of hydroxylating dehydrogenases [144]. The reduced flavin transfers the electrons to O_2, and, as another difference to flavin-dependent oxygenases, the resulting hydroperoxyflavin is not stabilized, leading to hydrogen peroxide formation. A remarkable feature of this oxidase family is that it favors the covalent attachment of the flavin cofactor [557].

Alcohol oxidases (AO, EC 1.1.3.13) are flavin-dependent enzymes, where FAD or FMN function as the primary hydride acceptor, and from which the excess reducing equivalents are shuttled to molecular oxygen [145,560,561]. Aliphatic alcohol oxidases from methylotrophic yeasts such as *Candida*, *Hansenula,* or *Pichia* sp. mostly accept primary alcohols as substrates, which are selectively oxidized to the corresponding aldehydes [540,562–565].

Likewise, glucose oxidase (GOX, EC 1.1.3.4) is a prominent flavoenzyme that catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone, utilizing molecular oxygen as an electron acceptor with simultaneous production of hydrogen peroxide [145,566–568]. In the reductive half reaction, the enzyme catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone, which is spontaneously and nonenzymatically hydrolyzed to gluconic acid. Subsequently, the FAD ring of GOX is reduced to $FADH_2$ [569]. In the oxidative half-reaction, the reduced enzyme is reoxidized by oxygen to yield H_2O_2 [570]. Usually, the formed hydrogen peroxide is then cleaved by catalase to produce water and oxygen. Its molecular weight ranges from 130 to 175 kDa [571]. This enzyme is receiving much attention due to its wide use in chemical, pharmaceutical, and food and beverage industries, as well as in clinical chemistry and biotechnology, mainly in novel applications for biosensors [572–574]. Microbial wild-type GOX is highly specific for β-D-glucose. GOX from ascomycetes is a dimeric glycoprotein composed of two identical polypeptide chain subunits which are covalently linked together *via* disulfide bonds [571,575]. The structure of *Penicillium amagasakiense* GOX shows each of its subunits to contain one mole of tightly bound, but not covalently linked, FAD moiety as cofactor [569,575]. This enzyme is glycosylated, with a mannose-rich carbohydrate content of nearly 11–13% [566]. The key conserved active-site residues of GOX from *P. amagasakiense* are Tyr73, Phe418, Trp430, Arg516, Asn518, His520, and His563 [569], being Arg516 the most critical amino acid for the efficient binding of β-D-glucose to the enzyme, while Asn518 contributed to a lesser extent. The aromatic residues Tyr73, Phe418, and Trp430 are important for the correct orientation of the substrate as well as for the maximal rate of its oxidation. His520 and His563 establish hydrogen bonds with the 1-OH of glucose during the reaction.

Pyranose oxidases (PyO, EC 1.1.3.10) are flavoproteins expressed in many lignin-degrading white rot fungi [576]. Unlike GOX, which acts on the anomeric carbon, PyO oxidizes in the presence of oxygen, glucose, and a number of common monosaccharides at their hydroxyl group in C-2, giving the corresponding 2-ketosugars and hydrogen peroxide. It is believed that these fungi use this transformation to produce H_2O_2 for their lignin-decomposing peroxidases [199]. The enzyme was first characterized in the 1960s [577], and since then important biotechnological applications have been developed in analytical as well as in synthetic carbohydrate chemistry.

PyO can also use quinones, complexed metal ions, and radicals as electron acceptors [199]. Some of these compounds are even better substrates for the enzyme compared with oxygen, suggesting that PyO can also play a direct role in the reduction of quinones during the process of ligninolysis [578]. Intracellular PyO from *Trametes multicolor* was purified from mycelia extracts and characterized by Haltrich and coworkers [579]. The native enzyme has a molecular mass of 270 kDa and is composed of four identical 68 kDa subunits, each one containing one covalently bound FAD as its prosthetic group.

In addition to the oxidation of the C–O bond to produce carbonyl compounds, oxidases are also well known as catalysts for the oxidation of C–N bonds, including examples in which these reactions occurs enantioselectively [533]. The most relevant oxidases catalyzing the oxidation of primary and secondary amines are flavoproteins, conducting the transfer of a hydride equivalent from the C–N bond to the flavin cofactor. Most of these AOs can be classified into two structural families: (i) the monoamine oxidase family (MAO) and (ii) the amino acid oxidase (AAO) family that comprises L-amino acid oxidases (LAAO, EC 1.4.3.2) and D-amino acid oxidases (DAAO, EC 1.4.3.3) [580]. The mammalian flavin-dependent MAO family includes MAO A and B (EC 1.4.3.4) [581–583] as the most interesting examples regarding synthetic applications. There are also flavin-dependent MAOs of fungal origin with increasing use in synthesis (see Chapter 4). Similar to copper-containing AOs, both flavin-dependent MAO A and B catalyze the oxidation of the primary amino groups of aromatic alkyl amines and are widely distributed in higher eukaryotes. The two isoforms of MAO (MAO A and MAO B) are separate gene products with 70% sequence identity, but exhibit overlapping substrate specificities in catabolism of endogenous amine neurotransmitters such as serotonin and dopamine, as well as a wide range of dietary amines and drugs [581]. Mammalian MAOs are bound to the outer mitochondrial membrane and have a FAD molecule covalently bound to the protein *via* an 8α-thioether linkage to a cysteinyl residue [584]. Although both enzymes have the same overall structure, MAO A has a more open active site, producing differences in substrate specificities for the two forms [581–583,585]. However, their catalytic mechanisms are proposed to be the same, and both have been discussed. Mechanism I involves a radical intermediate, while mechanism II proposes the nucleophilic attack of the amine substrate to the flavin C-4a position (Figure 1.28) [584,585].

Radical-based mechanisms for MAO are supported on studies with inhibitors containing cyclopropyl (or cyclobutyl) rings, since *trans*-2-phenylcyclopropylamine has been reported to be a time-dependent inhibitor for these enzymes [586]. Subsequently, Paech and coworkers showed that this compound is also a substrate, and that the inactivation results in covalent attachment of the inhibitor to the protein rather than to the covalently bound FAD [587]. These authors proposed that inhibition was due to nucleophilic attack of a protein thiol on the resulting cyclopropyl imine or its hydrolysis product. A number of other primary and secondary amines also inactivate the enzymes, through labeling of the protein or the flavin, or both [588]. These findings support a mechanism of inactivation involving one-electron transfers, although it was suggested that a protein rather than a flavin radical reacted with the inhibitor [589].

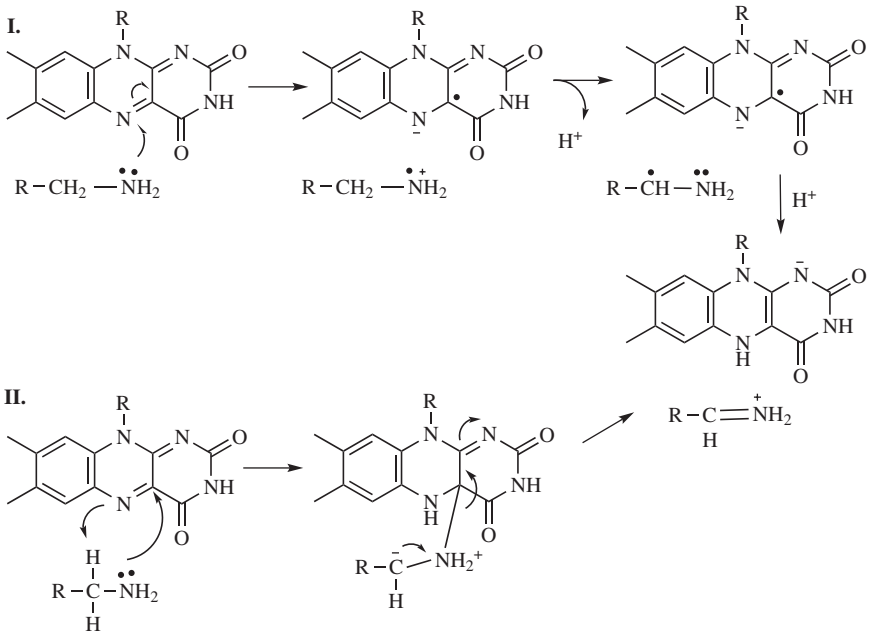

FIGURE 1.28 Proposed catalytic mechanisms for MAO A and B.

Mechanism II for the catalytic mechanism of MAO was developed by Edmondson et al., showing a nucleophilic mechanism for amine oxidation by MAOs and related enzymes [585]. A flavin-amine adduct forms prior to abstraction of the substrate hydrogen as a proton. However, this requires that no irreversible steps precede C–H bond cleavage, so that adduct formation must be reversible. Moreover, there is no obvious base in the active site of the enzyme to abstract a proton from the substrate [581]. This observation led to the mechanism II shown in Figure 1.28, in which the flavin N-5 is the base, with the proton abstraction occurring simultaneously as the substrate nitrogen adds to the flavin rather than in separate steps [585]. The pK_a for the neutral N-5 in reduced flavins has been estimated to be greater than 20 [590].

The other important family of flavin-dependent oxidases with relevant synthetic applications is the AAO family. D-Amino acid oxidases (DAAO) have been more studied and used in preparative applications than the L-selective enzymes [533]. DAAO has been employed for many years as an industrial biocatalyst in the commercial production of 7-aminocephalosporanic acid (7-ACA). The enzyme catalyzes the initial oxidative deamination of the D-amino acid side chain in cephalosporin C [591]. The D-amino acid oxidase family contains DAAO [592] and enzymes that oxidize glycine or *N*-methylated amino acids [593–597]. Thus, both MAO and DAAO families of AOs contain members that oxidize amino acids as well as primary and secondary amines. DAAO was first reported from kidney in 1935, and decades later became a model for the study of FAD-dependent oxidases [591,598]. More recently discovered bacterial glycine oxidases have similar structure to DAAOs from pig

kidney and yeasts, oxidizing D-alanine but not L-alanine [593,599], and are proposed to have the same mechanism of catalysis. The catalytic cycle involves a reductive half-reaction in which a hydride equivalent is transferred from the substrate to the flavin yielding the oxidized substrate bound to the reduced enzyme [580]. This step is energetically favorable for most substrates, thus enzyme reduction is effectively irreversible. The product then dissociates from the enzyme, which subsequently reacts with oxygen generating the oxidized enzyme, or else oxygen can react with the reduced enzyme before product dissociates, followed by product dissociation from the oxidized enzyme. The oxidation of an amine group involves the net loss of two electrons, as the C–N single bond is converted to a double bond, and of two protons, one from the carbon and one from the positively charged nitrogen. The ability of the flavin to accept one or two electrons and to form adducts with nucleophiles, in combination with the various possible sequences of loss of protons and electrons from the substrate, allows to propose a variety of catalytic mechanisms [580]. Through kinetic studies of pig kidney DAAO with a series of ring-substituted phenylglycines as substrates, it was concluded that the α-hydrogen of the substrate was removed as a proton [600]. Subsequently, it was proposed that oxidation of an amine or an alcohol could occur by nucleophilic attack of the heteroatom on the flavin C-4a position, followed by loss of the α-hydrogen as a proton (Figure 1.29) [601].

Later, Walsh et al. described the ability of DAAO to catalyze the elimination of HCl from β-Cl-alanine in addition to the normal oxidation of the substrate to β-Cl-pyruvate [602]. This was considered an additional evidence for proton removal to form a substrate carbanion in the initial step of catalysis, consistent with the proposed mechanism (Figure 1.30).

The ratio between oxidation and HCl elimination varied with the oxygen concentration, where higher amounts of O_2 favored the oxidation. To account for the effect of the oxygen concentration on the ratio between oxidation and elimination, it was necessary to propose the reaction of the carbanion intermediate with the flavin as a reversible step. The elimination reaction was subsequently studied with α-amino-β-Cl-butyrate, establishing that the hydrogen originally on the substrate α-carbon was found at the β-carbon in the α-ketoglutarate formed in the elimination reaction [580]. For this suggested mechanism, the tautomerization of the initial enamine to the imino acid and its hydrolysis to the corresponding ketoacid is required to occur in the active

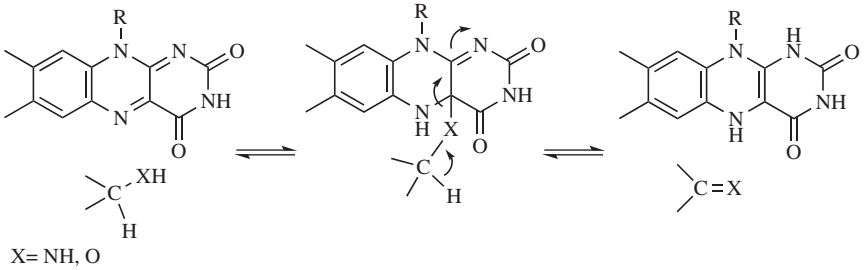

FIGURE 1.29 Proposed mechanism for the oxidation of amines (and alcohols) by flavin-dependent oxidases, on the basis of kinetic studies of pig kidney DAAO [601].

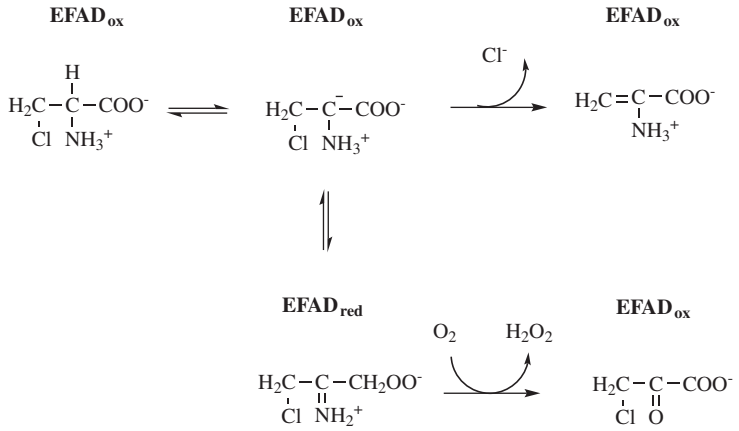

FIGURE 1.30 Proposed mechanism for DAAO-mediated oxidation of amines, consistent with the proton removal to form a substrate carbanion as initial step in the catalytic cycle.

site of the enzyme, in contrast to the behavior of other substrates, where the imino acid product dissociates from the enzyme before the ketoacid is formed [603].

An alternative mechanism was suggested accounting for both the effect of the oxygen concentration on the partitioning between elimination and oxidation, and the retention of the α-hydrogen on the β-carbon (Figure 1.31).

The amino acid substrate is oxidized to an imino acid by the normal catalytic mechanism. The reduced flavin can react with O_2 thus forming the oxidized enzyme, followed by dissociation of the β-Cl-imino acid. Alternatively, the reduced flavin can act as a hydride donor for an S_N2 reaction in which the chloride is the leaving group producing the oxidized enzyme and the β-Cl-imino acid. This mechanism accounts for both the oxygen dependence of the product, and the shift of the α-hydrogen to the β-carbon of the product. If the reduced flavin acts as a donor to the imino acid in the elimination, it is likely to act as a hydride acceptor from the imino acid in the normal reaction.

1.3.4 Peroxidases

Peroxidases are ubiquitous enzymes present in all domains of life, being found in the cytosol, in specific organelles, or are even secreted to the extracellular medium [604]. These enzymes use hydrogen peroxide (H_2O_2) or organic hydroperoxides (ROOH) as electron accepting cosubstrates, oxidizing a variety of compounds and do not require external addition of coenzymes for their regeneration [149,605]. They catalyze a wide variety of reactions under mild and controlled conditions. Among them, olefin epoxidations, sulfoxidation, halogenation, hydroperoxyde reduction, and allylic, propargylic, and benzylic hydroxylations (but no aliphatic hydroxylations) have been reported [37,606–608]. The vast majority of peroxidases are heme-enzymes, containing ferric protoporphyrin IX (protoheme) as prosthetic group in their active sites. In addition, there are several nonheme peroxidases which contain either

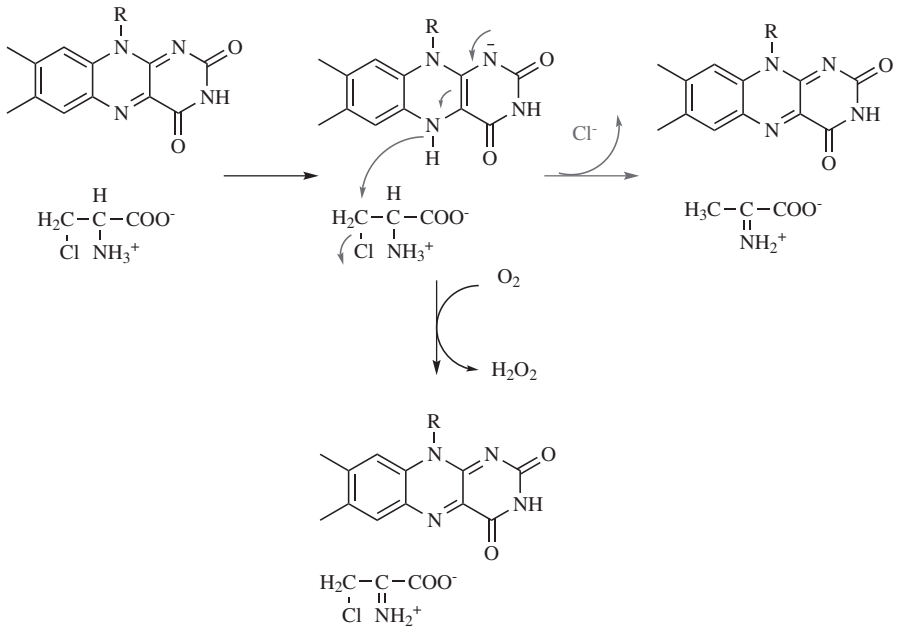

FIGURE 1.31 Proposed mechanism for the oxygen concentration-dependent amine oxidation versus HCl elimination in DAAO-mediated reactions.

other metals instead of iron (Mn, V, Se) such as vanadium haloperoxidases or manganese catalases [117,606], or specific metal-free prosthetic groups like the thiol peroxidases or the alkyl hydroperoxidases [609–611]. Peroxidases are grouped under EC 1.11.- (donor: peroxide oxidoreductases) in the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) system of enzyme classification [148,612]. Since the classification system is based on the catalyzed reactions, peroxidases with different molecular architecture and/or catalytic mechanism may be classified under the same EC number. For instance, the entry EC 1.11.1.10, corresponding to fungal chloride peroxidases, includes vanadium- (halide peroxidases) and heme-peroxidases (chloroperoxidase), and they are very different regarding their three-dimensional structure. However, peroxidases have also been classified according to other characteristics, such as structural properties and sequence information [611]. Concerning the heme-containing peroxidases, this has led to a classification in two main superfamilies: (i) the peroxidase-cyclooxygenases (animal or mammalian peroxidases) and (ii) the nonanimal peroxidases (plant peroxidases), which include most of the heme-dependent peroxidases. In addition, according to the sequence, host organism, and physiological function, plant peroxidases comprise three main classes, I, II, and III (see Section 1.2.1) [117,609,613–618]. Within the peroxidase-cyclooxygenase superfamily, which includes lactoperoxidase (LPO), thyroid peroxidase, and myeloperoxidase, similar subgrouping according to physiology and enzyme function was established [619]. However, there are already

a number of new fungal heme-containing peroxidases which do not fit into the two previous superfamilies: (i) the synthetically relevant heme-thiolate peroxidases, comprising chloroperoxidase from *Caldariomyces fumago* (CPO, EC 1.11.1.10) and aromatic peroxygenases from basidiomycetes; and (ii) the dye-decolorizing peroxidases (DyPs) from fungi and eubacteria [117,611,616,620–623]. Peroxidases are often named according to their natural source, such as horseradish peroxidase (HRP, EC 1.11.1.7) from *Armoracia rusticana*, LPO from bovine milk, or myeloperoxidase (MPO) from human leukocytes. They can also be named according to their natural substrates, namely cytochrome C peroxidase (CcP, EC 1.11.1.5), chloroperoxidase from *C. fumago* (CPO, EC 1.11.1.10), or lignin peroxidase (LiP, EC 1.11.1.14).

The most frequently used peroxidases in organic synthesis are chloroperoxidase (CPO) from *C. fumago* and peroxidase from *A. rusticana* root (HRP). Chloroperoxidase is the most versatile peroxidase, and also behaves as a monooxygenase in sulfoxidations with molecular oxygen and external reducing agent, with substantial increase in enantioselectivity and stability [624]. The classical peroxidase-catalyzed reaction consists of a one-electron oxidation of the substrate with a peroxide as electron acceptor [144]. Heme-dependent peroxidases in turn, also catalyze other oxygen transfer reactions such as olefin epoxidations, sulfoxidations, or hydroxylations [37,606–608]. Such reactions are two-electron oxidations, in which peroxide serves as oxygen donor and electron acceptor, and one molecule of water (or alcohol

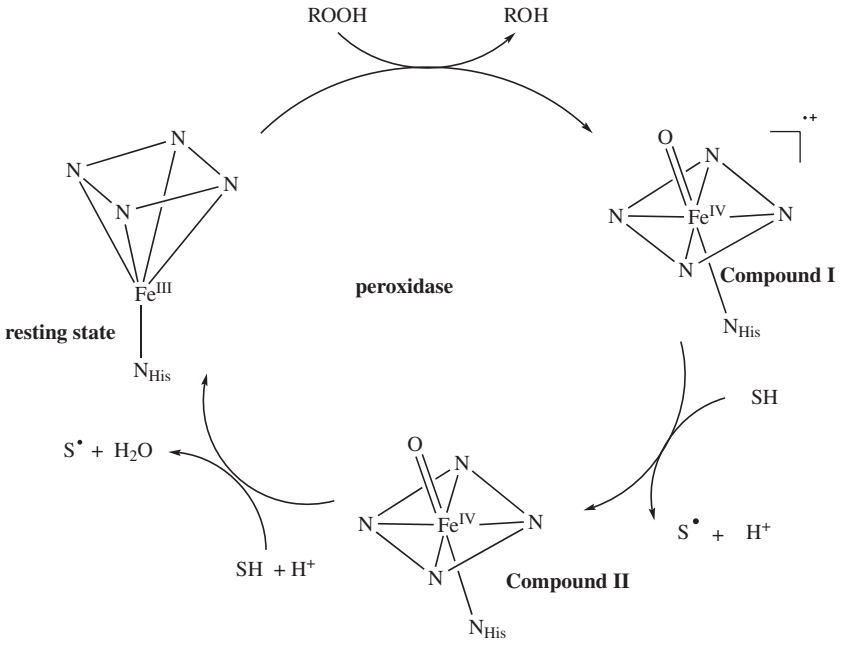

FIGURE 1.32 Catalytic cycle of heme-peroxidases. SH: substrate.

in case of organic peroxide driven reactions) is produced as coproduct. In these oxygen transfer reactions, heme-dependent peroxidases exhibit more typical reactivity for CYP450 than of classical peroxidases, which catalyze oxidative dehydrogenations *via* one-electron processes. This class of peroxidases are not only functionally but also structurally related to P450 monooxygenases, which are also able to catalyze substrate oxyfunctionalizations with hydrogen peroxide as oxygen source in the so-called peroxide shunt pathway. The catalytic mechanism of heme-peroxidases for the classical peroxidase reaction has been studied using HRP as model (Figure 1.32) [606,625].

In heme-dependent peroxidases, hydrogen peroxide enters the catalytic cycle at the hydroperoxo-stage coordinating as a hydroperoxyl anion to Fe(III) and then leading to the pivotal intermediate compound I. In addition to this classical reaction, oxygen transfer from oxidized peroxidase to the substrate can also take place, provided that the heme iron is accessible for the substrates (see Chapter 5, Section 5.2). The fifth ligand of the iron is a histidine, except in CPO, which belongs to the group of heme-thiolate peroxidases [110,626]. With respect to oxygen transfer reactions, this peroxidase with iron ligated to cysteine, similar to P450s, is the most versatile enzyme of the known peroxidases (see Chapter 5, Section 5.2.1.3)

1.4 CONCLUDING REMARKS

As exposed in this chapter, there is a significant diversity of enzymes, reactions, and processes occuring in Nature. Many of these performances may be useful for industrial catalytic processes, once key-aspects of the reactions (e.g., cofactor regeneration) are properly optimized. In a nutshell, enzymatic redox reactions can be gathered in three different groups: (i) dehydrogenase-catalyzed, nicotinamide cofactor-dependent reactions; (ii) oxidations at the expense of molecular oxygen, catalyzed by mono- or dioxygenases, (also nicotinamide cofactor-dependent biocatalysts); (iii) peroxidations, which require hydrogen peroxide and are catalyzed by peroxidases. In Chapters 3–6, different applications of these type of enzymes have been discussed in depth.

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