1

BASIC MITOCHONDRIAL PHYSIOLOGY IN CELL VIABILITY AND DEATH

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1. Introduction 3
   1.1. Historical background 3
   1.2. Morphology 4
   1.3. Structure and compartmentation 6
2. Oxidative phosphorylation 7
   2.1. General principles 7
   2.2. Respiratory chain as a proton pump 8
   2.3. Mitochondrial ATPase/ATP synthase and energy coupling 12
   2.4. Coupling and uncoupling; reversed electron transport 15
   2.5. Mitochondrial carriers 17
3. Production of reactive oxygen species 17
4. Calcium signaling 23
5. Mitochondria and cell death 25
6. Concluding remarks: mitochondria as a pharmacological target 31

1. INTRODUCTION

1.1. Historical Background

Mitochondria were described by histologists and cytologists during the second half of the nineteenth century as minute intracellular granules of various sizes and...
shapes. The discovery by Leonor Michaelis in 1898 that they could be stained with the reduction–oxidation dye Janus green was probably the first indication that they were sites of intracellular redox processes. This notion was reinforced by an observation of Otto Warburg, who found in 1913 oxygen consumption by a particulate fraction obtained from tissue dispersions (and he received a Nobel prize in 1931). The name *mitochondrion* was coined from two Greek terms, μιτός (*mitos*, a thread) and χονδρίον (*khondrion*, a grain), which best characterized the microscopic appearance of these structures.

However, it was not until Albert Claude isolated relatively pure mitochondria in substantial amounts from tissue homogenates by differential centrifugation (1940) that progress in elucidating their importance for cell functions accelerated. In the 1930s, when Hans Krebs showed that the tricarboxylic acid cycle localized to mitochondria, they were recognized as important chemical factories (he received a Nobel prize in 1953). The following years saw the discovery of oxidative phosphorylation, and mitochondria emerged as the main source of cellular ATP [1,2]. However, the modern concepts of oxidative phosphorylation (Nobel prize to Peter Mitchell in 1978) and molecular mechanisms of oxidative phosphorylation (Nobel prize to Paul D. Boyer and John E. Walker in 1997) were formulated later [2–4].

With the discovery of rotating ATPase/ATP synthase [5] (see Section 2), the era of fundamental discoveries related to mitochondria as cellular energy transformers seemed to come to an end. More recently, mitochondriology has undergone a renaissance and is now a focus of intense interest in a wide variety of life sciences. First, the discovery that defects in mitochondrial DNA (mtDNA) are the basis for mitochondrial diseases launched the new field of mitochondrial medicine [6]. Second, mitochondria were recognized to play a major role in initiation and execution of programmed cell death (apoptosis) [7,8]. Among other new areas of study are the mitochondrial theory of aging [9] and mechanisms of intracellular signaling [10]. Mitochondria are also targets, either primary or secondary, of numerous therapeutic and toxic xenobiotics [11].

1.2. Morphology

Our knowledge of the inner structure of mitochondria is based primarily on electron microscopic examination of glutaraldehyde- and osmium tetroxide–fixed preparations of whole cells or tissues and of isolated particles (see also Chapter 23). Mitochondria are composed of two membranes that separate two compartments, the intermembrane compartment and the inner compartment, filled with the mitochondrial matrix (Figure 1). The outer membrane is usually smooth and forms a boundary separating the mitochondrion from the cytosol. The inner membrane forms multiple invaginations into the matrix compartment, the cristae. Depending on the tissue, the density of cristae varies from quite scarce, as in liver mitochondria, to tightly packed, as in muscle mitochondria, where the inner compartments are densely filled with cristae. This usually correlates with the roles played by mitochondria in different tissues.
INTRODUCTION

Figure 1 Electron micrograph of mitochondria in pancreatic centroacinar cell. Bottleneck-like contacts between the intracristal space and the intermembrane space are indicated by arrows. (Reproduced from Tzagoloff [2], with permission from the author and Springer Science and Business Media.)

[i.e., whether they function mainly as energy transducers or as “chemical factories” (e.g., urea production in liver mitochondria)]. The intracristal compartments form a continuum with the intermembrane space. However, due to narrowness and elongation of the cristae and, quite often, a bottlenecklike shape of the connections between the cristae and the intermembrane compartment, free mixing of the contents of both spaces may be hindered (see the discussion of cristae junctions in Chapter 23).

In many tissues, separate mitochondria observed by electron microscopy may, in fact, constitute fragments of larger, branched structures. In extreme cases, as in some protozoan or yeast species, it is proposed that the cell contains a single giant mitochondrion whose multiple fingerlike branches may, in thin sections, look like separate mitochondria. In metazoans, mitochondria stained with fluorescent dyes and viewed in a light microscope often form elongated threadlike structures (Figure 2), described by some authors as the mitochondrial network. Moreover, in live cells these structures are not only in constant motion but also split and fuse again. This phenomenon, observed first decades ago, has attracted more attention in recent years, as it appeared to be related, among other factors, to the mitochondrial energy state and genetic status and to play a role in the programmed death of cells (apoptosis; see Section 5).
1.3. Structure and Compartmentation

Despite this dynamic situation, the four intramitochondrial entities described above (i.e., the outer membrane, the intermembrane compartment, the inner membrane, and the matrix compartment) retain their distinct composition and characteristics. Both membranes are formed of phospholipid bilayers with multiple integral and peripheral proteins, many with transporting and enzymatic functions. The outer membrane contains the mitochondrial porin or voltage-dependent anion channel (VDAC) that enables the membrane to function as a molecular sieve by allowing compounds of up to 5000 Da to pass freely while preventing diffusion of larger molecules. It has to be mentioned that despite its name, VDAC is only partly selective toward anions, and it also allows more-or-less free diffusion of cations and uncharged molecules. Thus, the composition of the intermembrane compartment is similar to that of the cytosol as far as low-molecular-weight compounds are concerned. Among its lipidic compounds, the outer membrane contains cholesterol, in contrast to the inner mitochondrial membrane, which is essentially cholesterol-free. This property enables solubilization of the outer membrane by compounds complexing cholesterol, such as digitonin, thus making it possible to obtain mitochondria stripped of the outer membrane, called mitoplasts.

Due to the impermeability of the outer membrane to large-molecular-weight compounds, the enzyme composition of the intermembrane compartment differs considerably from that of the cytosol. It is mainly the site of transphosphorylation reactions. An important example is the formation of ADP from AMP and ATP, catalyzed by adenylate kinase:

\[
AMP + ATP \rightleftharpoons 2ADP
\]  

(1)
The inner mitochondrial membrane contains the entire respiratory chain plus the ATP synthase complex. Due to operation of the respiratory chain coupled with proton pumping, the inner membrane is also a site of high voltage difference on both sides, ranging up to 180 mV over the membrane thickness of about 100 nm. This capacitance reflects the extremely high insulating properties of the phospholipid membrane bilayer. On the other hand, the inner mitochondrial membrane contains several specific transporters for anionic metabolites, including respiratory substrates, inorganic phosphate, ADP, and ATP. A characteristic feature of most transporters is that they operate as exchange carriers (e.g., transporting dicarboxylic acids in exchange for phosphate or exchanging ATP for ADP, among others; see Section 2.5).

The inner compartment encompasses the mitochondrial matrix. This dense solution of enzymatic proteins, coenzymes, metabolites, and inorganic ions is the site of the citric acid cycle (the Krebs cycle), which provides reducing equivalents to the respiratory chain. The matrix also contains the mitochondrial genome responsible for the limited genetic autonomy of the mitochondrion. Mitochondrial DNA (mtDNA), like bacterial DNA, is circular in shape and contains 37 genes. The mtDNA of the prokaryotic type is one of the arguments supporting the endosymbiotic concept of mitochondria origin according to which these organelles developed from prokaryotic organisms that invaded precursors of the present eukaryotes [12].

2. OXIDATIVE PHOSPHORYLATION

2.1. General Principles

Approximately 95% of ATP formation in animal cells with aerobic type of metabolism occurs by oxidative phosphorylation (OXPHOS), and mitochondria can be considered as cellular powerhouses converting energy released during substrate oxidation into a form available for cellular processes. Therefore, although mitochondria are a site of many biosynthetic and metabolic processes, OXPHOS is paramount.

OXPHOS consists of two functionally independent processes: oxidation of reduced substrates (expressed as respiration or oxygen consumption) and phosphorylation of ADP by inorganic phosphate. The latter, energy-consuming process occurs at the expense of energy released during the former. Thus, the two elements of oxidative phosphorylation are coupled to each other obligatorily. The mechanism of this coupling results from specific properties of the inner mitochondrial membrane, which is the location of oxidative phosphorylation. From a bioenergetic perspective, the most important feature of the inner mitochondrial membrane is its composition, and as a consequence, its extremely selective permeability to a variety of substances. In comparison to other membranes of an animal cell, the inner mitochondrial membrane contains a much higher proportion of proteins (approximately 80%) and only 20% phospholipids. Among the latter, 10% is cardiolipin, a unique mitochondrial phospholipid with four acyl chains.
In contrast, the proportion between proteins and lipids in the outer mitochondrial membrane is approximately 1:1, which is more typical for most other cellular membranes.

In contrast to the outer membrane, the inner mitochondrial membrane can be crossed passively by only a few compounds, such as weak acids (e.g., acetic acid), water-dissolved gases (oxygen, ammonia), and lipophilic compounds. Electrically charged and hydrophilic compounds such as carboxylic anions (including respiratory substrates) and inorganic ions, are unable to pass the inner membrane without participation of specialized transporting proteins (see Section 2.5). High resistance of the inner mitochondrial membrane to protons is crucial for OXPHOS.

From a functional point of view, the oxidative phosphorylation machinery consists of two proton-pumping systems capable of proton translocating across the inner membrane from the mitochondrial matrix to the intermembrane compartment, located in a highly H\(^+\)-impermeable lipidic core of the membrane. One of these pumps is the respiratory chain as a whole, and the other is the mitochondrial ATPase. As, under physiological conditions, it catalyzes ATP formation at the expense of energy delivered during the respiration, it is also defined as ATP synthase. This name clearly depicts the real function of this enzyme in oxidative phosphorylation. Taken together, ATP formation catalyzed by mitochondrial ATPase is driven by the mitochondrial inner membrane electrochemical proton gradient (mitochondrial protonmotive force, \(\Delta p\)) built up during respiration. This statement is the most condensed summary of Peter Michell’s principle of the chemiosmotic concept of oxidative phosphorylation (Figure 3; for a comprehensive overview, see [3]).

2.2. Respiratory Chain as a Proton Pump

Mitochondrial respiratory chain catalyzes electron transfer from the reduced donors (NADH and FADH\(_2\)) to molecular oxygen (O\(_2\)). The final product of this pathway is water. Because of a large redox potential difference between electron donors and the final electron acceptor (about 1.10 and 0.90 V for NADH and FADH\(_2\) as electron donors, respectively), which is a reflection of the displacement of the system from equilibrium, electron flow along the respiratory chain is accompanied by a significant decrease in the Gibbs potential (i.e., release of a large amount of free energy). Under physiological conditions, a large proportion of this energy is used to pump protons across the inner mitochondrial membrane from the matrix to the intermembrane space. The remaining energy is dissipated as heat. The proportion of energy utilized to generate the electrochemical proton gradient versus that dissipated as heat depends on the type of tissue and its physiological state.

The unequal distribution of protons between the two sides of the inner mitochondrial membrane results in the generation of an electrochemical potential across it consisting of two components: a potential that reflects unequal distribution of electrical charges (\(\Delta \Psi\)), and the chemical potential resulting from an unequal distribution of chemical entities, mainly protons (more precisely,
Figure 3  Schematic representation of the chemiosmotic concept of energy coupling. The inner mitochondrial membrane contains the respiratory chain that operates as a proton pump by translocating protons from the inner to the outer side of the membrane, thus forming the electrochemical proton gradient (the protonmotive force, $\Delta p$) composed of the electric component ($\Delta \Psi$, positive outside) and the chemical component ($\Delta \text{pH}$, acidic outside). $\Delta p$ then drives protons backward through the $F_1F_0$ complex (ATP synthase), becoming the driving force of ATP synthesis. The $F_1F_0$ complex can also operate in the reverse direction (as mitochondrial ATPase), hydrolyzing ATP and ejecting protons to the outside, thus building $\Delta p$.

Hydrated hydrogen ions $H_3O^+$ expressed as $\Delta \text{pH}$:

$$\Delta p = \Delta \Psi - \Delta \text{pH}$$  \hspace{1cm} (2)

In fully energized mitochondria, $\Delta \Psi$ amounts to 180 to 200 mV, negative inside (the matrix side of the inner membrane is called the N-side, for “negative,” and the external side is designated as the P-side, for “positive”). The hydrogen ion concentration difference in animal mitochondria is usually about 0.5 pH unit, which corresponds to 30 mV. Because, in energized mitochondria, pH is higher inside mitochondria than outside, the pH difference is formally negative. Thus, the total protonmotive force of energized mitochondria may reach a value of 210 to 230 mV. Summing up, the mitochondrial electrochemical membrane potential may be regarded as an intermediate source of energy that is released during respiration and is made available for other, energy-consuming processes, such as ATP synthesis and metabolite transports across the inner membrane.
As $\Delta p$ is a consequence of proton pumping across the inner membrane during electron flow along the respiratory chain, an important question arises concerning the efficiency of the mechanisms transforming energy released during chemical reactions into the electrochemical proton gradient. The exact stoichiometry between electron flow and proton pumping is still debated, although the most widely accepted figure is that 10 $H^+$ are translocated from the mitochondrial matrix to the intermembrane space for each pair of electrons transported from NADH to oxygen. In the case of FADH$_2$, which donates two electrons to the respiratory chain one step downstream, at complex II, only six protons are extruded from the matrix compartment.

Stoichiometry does not, however, define the efficiency of ATP formation in relation to the oxygen consumed. It must be stressed that variable amounts of $\Delta p$ can be dispersed as a passive proton leak that bypasses ATP synthesis and other energy-consuming processes driven by $\Delta p$, such as ion and metabolite transport. Therefore, the actual stoichiometry of oxidative phosphorylation is usually expressed as the P/O ratio, the ADP/O ratio, or more generally, the $P/2e^-$ ratio. These ratios express the number of molecules of phosphate used for ADP phosphorylation in terms of the number of oxygen atoms consumed or the number of electron pairs transported along the respiratory chain [3].

The mitochondrial respiratory chain is composed of more than 85 proteins assembled in four complexes. Complexes I (NADH–ubiquinone oxidoreductase), III (ubiquinol–cytochrome c oxidoreductase), and IV (cytochrome c oxidase) are located in the inner mitochondrial membrane as integral proteins, whereas complex II, comprising succinate dehydrogenase, which catalyzes one of the steps of the citric acid cycle, is attached to the inner surface of the inner membrane. These enzymatic complexes are connected functionally by diffusible electron acceptors and donors: ubiquinone/ubisemiquinone/ubiquinol and oxidized/reduced cytochrome c. Figure 4 shows the sequence of reactions that comprise the mitochondrial respiratory chain.

Complex I (NADH–ubiquinone oxidoreductase) catalyzes the oxidation of reduced nicotinamide nucleotides concomitantly with the reduction of ubiquinone (UQ) to ubiquinol (UQH$_2$). This reaction is coupled to the pumping of four protons from the matrix to the intermembrane space per pair of electrons transferred from NADH. Complex I is the largest one within the respiratory chain, consisting of 43 polypeptides. Its redox center contains flavin mononucleotide and a few (six or seven) iron–sulfur centers. Inhibition of this complex, which prevents electron flow to ubiquinone and therefore causes a large accumulation of NADH, may lead to an enhanced formation of reactive oxygen species.

As an electron carrier, ubiquinone accepts electrons from both complex I and succinate dehydrogenase, which is the essential part of complex II. Complex II is located at the internal side of the inner membrane and is the only respiratory complex encoded completely by nuclear DNA. It catalyzes oxidation of succinate to fumarate in the tricarboxylic acid cycle, with concomitant reduction of FAD and subsequent reduction of ubiquinone. This enzyme is composed of four subunits. One of them, containing FAD, participates in the oxidation of succinate.
Another, the extramembrane subunit, contains three Fe–S clusters that transport electrons to the next two subunits, which are internal membrane proteins, and then to ubiquinone. Electron transfer from succinate to UQ is not coupled to H⁺ translocation across the inner membrane.

Ubiquinone can also be reduced in the reaction catalyzed by sn-glycero-phosphate dehydrogenase (bound to the outer surface of the inner membrane) and by the electron-transferring flavoprotein (ETF), a soluble enzyme in the matrix that mediates electron transfer in fatty acid oxidation (not shown in Figure 4). The enzymatic activity of these FAD-containing oxidoreductases, similar to that of complex II, is not directly connected to ΔΨ generation.

The next reaction of the respiratory chain is the electron transfer from ubiquinol to cytochrome c, catalyzed by complex III, ubiquinol–cytochrome c oxidoreductase, also known as bc₁ complex. Complex III is a dimer consisting of 11 subunits per monomer. It contains a few redox groups, including a 2Fe–2S center (Rieske protein), and three heme molecules (two b-type cytochromes and one cytochrome c₁). The mechanism of complex III activity includes the Q-cycle, in which two-step oxidation of ubiquinol to ubiquinone occurs, with transient formation of ubisemiquinone. This mechanism allows translocation of four protons from the matrix to the intermembrane space. It is noteworthy that ubisemiquinone, being a free radical, may enhance superoxide radical formation via autoxidation. This process is especially efficient under conditions of high ΔΨ that prevent electron flow between heme molecules in the Q-cycle, thereby increasing the half-life of ubisemiquinone (see Section 3).

Finally, complex IV (cytochrome c oxidase) catalyzes sequential transfer of four electrons from reduced cytochrome c to molecular oxygen, forming two molecules of water. Complex IV is composed of 13 subunits, but only two of them (subunits I and II) are of high relevance to catalysis. Subunit II has a redox
center containing two copper atoms (CuA) clustered with a sulfur atom that undergoes one electron redox process. Subunit I comprises two heme groups (heme $a$ and heme $a_3$) and one copper atom (CuB). Complex IV is the least efficient $\Delta p$-generating proton pump in the respiratory chain. Calculating based on two electrons transferred to oxygen, only two protons are extruded to the intermembrane space. Other two protons combine with the oxygen atom and two electrons to produce water. Complex IV makes a relatively small contribution to generation of the mitochondrial protonmotive force despite large Gibbs free-energy. In contrast to reactions catalyzed by complexes I, II, and III, oxygen reduction by cytochrome $c$ is irreversible.

2.3. Mitochondrial ATPase/ATP Synthase and Energy Coupling

Mitochondrial ATPase (also called complex V, although it is not part of the respiratory chain) is a large protein complex. Negative staining techniques of electron microscopy reveal its structure as “mushroom-like” particles attached to the matrix side of the inner membrane. The head piece of this structure is attached to the membrane by a “stem” embedded in the lipidic phase of the membrane (Figure 5). The head piece, designated by Efraim Racker as coupling factor 1 (abbreviated $F_1$), has a molecular mass of 370 kDa, whereas the stem, of 160 kDa molecular mass, is identified as Racker’s coupling factor $O$ (sensitive to oligomycin, abbreviated $F_O$).

The catalytic mechanism of ATP synthase exploits the mitochondrial $\Delta p$ as a source of energy to displace the mass-action ratio for ADP phosphorylation by 7 to 10 orders of magnitude from equilibrium. During oxidative phosphorylation, protons diffuse down the concentration gradient from the intermembrane space

**Figure 5** Coupling factor 1 ($F_1$) visualized by negative staining. Numerous mushroom-like structures are visible attached to the inner side of inner membrane fragments of a disrupted rat liver mitochondrion. The diameter of the spherical “head” is about 10 nm, and the length of the “stalk” is 5 nm. (Electron micrograph by P. Wlodawer.)
into the matrix through the proton channel formed by the F$_O$ subunit. This proton current across the inner membrane is accompanied by a decrease in Gibbs free energy, which drives reversal of ATP hydrolysis. The catalytic activity of the enzyme is associated with the F$_1$ subunit, which hydrolyzes ATP if separated from F$_O$. ATP formation from ADP and inorganic phosphate in a protein-free solution is negligible because of the extremely low equilibrium constant for this reaction. Phosphorylation of ADP bound to F$_1$, although still very low, is detected even in the absence of $\Delta \varphi$, suggesting a slight increase in the equilibrium constant when reactants are bound to the F$_1$ catalytic center. Such observations indicate that the important energy-consuming step of oxidative phosphorylation is release of ATP from the enzyme active site. In fact, the energy of the proton electrochemical gradient is not utilized directly for combining ADP and inorganic phosphate but, rather, to constrain conformational changes of the catalytic subunits that dictate ADP, P$_i$, and ATP binding affinity and steric interaction.

F$_O$ is composed of three types of proteins, called subunits a, b, and c, with the first two encoded by mtDNA. The central channel of F$_O$ is formed by 10 c subunits organized in a symmetric ring traversing the inner membrane. Subunit a is connected asymmetrically with the external surface of the ring and subunit b, which extends from the membrane, connecting the transmembrane portion of F$_O$ with a distant subunit of the F$_1$ particle. Thus, F$_O$ forms the H$^+$-selective, oligomycin-sensitive, channel.

F$_1$ consists of five types of subunits: $\alpha$, $\beta$, $\gamma$, $\delta$, and $\varepsilon$, assembled with the stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$. Subunits $\alpha$ and $\beta$ are positioned alternately around subunit $\gamma$, forming a caplike structure containing three $\alpha\beta$ dimers. Subunit $\gamma$ forms a stalk that connects the cluster of $\alpha$ and $\beta$ subunits with the $c_{10}$ ring of F$_O$ [5] (Figure 6).

Each of the three catalytic centers of ATP synthase located on the three $\beta$ subunits is able to assume three different conformations, varying in their affinity for substrates (ADP and P$_i$) and product (ATP). Conformation O (for “open”) is characterized by low affinity to ATP; conformation L loosely binds ADP and P$_i$; and conformation T tightly binds ADP and P$_i$, leading to ATP formation. In energized mitochondria, protons flowing into the mitochondrial matrix compartment via the membrane-embedded F$_O$ sector force subunit $\gamma$ to rotate, while subunit b forms a stator, holding subunits $\alpha$ and $\beta$ stationary. For one complete turn of 360°, 10 protons must return to the matrix (i.e., one H$^+$ for one subunit c) [3]. Simplifying somewhat, one can assume that rotation by 120° results in a switch from one conformation to another. Thus, one subunit $\beta$, being at conformation O, changes its structure to conformation L; another subunit $\beta$, originally at conformation L, is transformed to conformation T; and a third subunit $\beta$, being at conformation T, returns to conformation O (Figure 7). As result, one full revolution of subunit $\gamma$ results in a complete cycle, in which three molecules of ATP are released. These sequential alterations of subunit $\beta$ conformation are elicited mechanically by rotation of the asymmetrically oriented subunit $\gamma$ within the
Subunit structure of mitochondrial ATPase/ATP synthase. Subunits c of $F_0$ are assembled as a ring plunged into the inner membrane. They allow protons to return to the mitochondrial matrix. Transient and sequential protonations of each of the 10 c subunits causes a clockwise rotation (when viewed from the membrane side) of subunit $\gamma$, driving a cycle of conformational changes of the $\alpha_3\beta_3$ assembly of $F_1$. Full 360° rotation requires 10 protons to pass across the inner mitochondrial membrane. This allows for phosphorylation of three molecules of ADP. OSCP (oligomycin sensitivity-conferring protein), together with subunits a and b, comprises a stator that prevents the $\alpha_3\beta_3$ assembly to rotate together with subunit $\gamma$. Note that the OSCP subunit is distant from $F_0$ and is not the oligomycin-binding site. However, it makes a link between subunit b and the $\alpha_3\beta_3$ assembly and prevents the latter from undergoing conformational changes in the presence of oligomycin. (Drawing by M. R. Wieckowski.) (See insert for color representation of figure.)

$\alpha_3\beta_3$ head-piece sector (Figure 6). Thus, mitochondrial $F_1F_0$-ATPase, equivalent to ATP synthase, represents an interesting example of a mechanochemical catalytic assembly, a “nanomotor” [14].

Mitochondrial ATP synthesis coupled to inwardly directed proton flux is fully reversible, meaning that ATP hydrolysis, catalyzed by the same enzymatic assembly, results in proton pumping in the reverse direction: from the matrix compartment out to the intermembrane space (and further on to the cytosolic compartment). Thus, under conditions of low $\Delta\rho$, cytosolic ATP (e.g., formed by glycolysis) is hydrolyzed to ADP and $P_i$ with a concomitant restoration of $\Delta\rho$. Applying ingenious microtechniques to a fluorescently labeled isolated $F_1$ sector immobilized on a coverslip, it was possible, using fluorescence microscopy, to observe rotation of subunit $\gamma$ under conditions of ATP hydrolysis [15]. Moreover, by attaching a magnetic bead to subunit $\gamma$ and applying a rotating magnetic field, researchers succeeded in obtaining the formation of minute but detectable amounts of ATP [16].
2.4. Coupling and Uncoupling; Reversed Electron Transport

As discussed above, most of the reduction–oxidation (redox) reactions of the electron transport system are reversible. Tight coupling of mitochondrial OXPHOS is therefore reflected by the reversibility of ATP synthesis/hydrolysis and transmembrane proton fluxes, and at least partial reversibility of electron flow in the respiratory chain and Δp formation. Indeed, electrons from ubiquinol can be transported “uphill” (i.e., against the redox potential) to complex I and on to NAD⁺ at the expense of Δp. The best known example is reduction of NAD⁺ to NADH by succinate. This process is termed reversed electron transport. Although the reversed electron flow can be observed in energized isolated mitochondria, its role within intact cell under physiological conditions remains unclear.
Under conditions of excess respiratory substrate and O\textsubscript{2} but no ADP and/or P\textsubscript{i}, \(\Delta p\) increases and becomes a limiting factor for electron flow. Such a condition is defined as the resting state or state 4 respiration and is characterized by very low oxygen uptake and is readily obtained in isolated mitochondria. Low O\textsubscript{2} uptake in these conditions is limited by a slow dissipation of \(\Delta p\). Such proton leak is due to a variety of factors, including weak uncoupling by nonesterified long-chain fatty acids that cross the inner mitochondrial membrane passively in undisassociated form, only to be transported in the opposite direction as anions by the adenine nucleotide carrier and other substrate carriers [17,18]. Futile cation fluxes and flickering of the unspecific permeability transition pore (see Section 5) also contribute to proton leak.

In contrast, in the presence of excess ADP and P\textsubscript{i}, mitochondria respire at the maximum rate, which is limited only by the rates of ATP synthesis and ATP/ADP exchange across the inner mitochondrial membrane (assuming that P\textsubscript{i} transport is not limiting). Such a metabolic condition is called the active state or state 3. Under these conditions, the energy of the electron flow along the respiratory chain is maximally utilized for ATP synthesis.

Under experimental conditions, the inner mitochondrial membrane can be made fully permeable to protons. This can be achieved by disrupting the membrane mechanically or using chemicals that can transfer protons across the phospholipid phase of the membrane. Such protonophores are typically lipophilic weak acids that can cross the lipid bilayer passively in both protonated and deprotonated forms. Most commonly used are 2,4-dinitrophenol (DNP), carbonyl cyanide \(m\)-chlorophenylhydrazone (CCCP), and carbonyl cyanide \(p\)-trifluoromethoxyphenylhydrazone (FCCP). The rate of respiration in the uncoupled state (also described as state U) is essentially limited by the efficiency of the respiratory chain and is usually equal to, or somewhat higher than, that in active state 3.

Physiological uncoupling is characteristic for some tissues, such as thermogenic brown adipose tissue, also called “brown fat,” present in neonatal mammals, including humans, and in mammals that hibernate. Mitochondria of this tissue may become almost completely uncoupled, due to the presence of a specific inner membrane protein, the uncoupling protein (UCP1), which enables passage of protons. The mechanism of this intrinsic property of UCP1 is similar to that described above for the adenine nucleotide carrier: namely, cycling of nonesterified fatty acids [19]. As a result, energy produced by the electron flow in brown adipose tissue mitochondria is not captured in the form of \(\Delta p\) and utilized for ATP synthesis, but rather, dissipated as heat [20].

Homologs of UCP1 have recently been identified in other tissues: heart, skeletal muscle, and brain and termed UCP2, UCP3, UCP4, and UCP5. They are present in minute quantities and contribute only slightly to the inner membrane permeability. It is hypothesized that their physiological roles include protection against free-radical formation [19].
2.5. Mitochondrial Carriers

Although mitochondrial membrane potential is used primarily to drive ATP synthesis, it is also used to drive other processes, including importation of ADP, inorganic phosphate, and respiratory substrates, and to maintain ion gradients. Moreover, ATP and other metabolites formed within mitochondria are exported to the cytosol. For example, liver mitochondria release malate or phosphoenolpyruvate to support cytosolic gluconeogenesis, and citrulline for cytosolic urea synthesis. As mentioned earlier, the inner mitochondrial membrane is impermeable to the majority of substances, including metabolites, phosphate, and inorganic ions. Hence, translocation into and out of the matrix is possible only via specific transmembrane carriers and channel-forming proteins. Some of these transported substances are accumulated within mitochondria, or released into the cytosol, against a concentration gradient, a process requiring energy obtained from $\Delta p$H or $\Delta \Psi$ [3]. Mitochondrial transporting mechanisms can be divided into the following four categories:

1. Electroneutral exchange driven by $\Delta p$H. For example, mitochondria accumulate inorganic phosphate, which is exchanged for $\text{OH}^-$ via the $P_i$/OH$^-$ antiporter, which is equivalent to the $P_i$/H$^+$ symport. Another example of such transport is the electroneutral exchange of a cation for a proton (e.g., $\text{Na}^+$/H$^+$). In energized mitochondria (alkaline inside) this exchange will favor the efflux of cations.

2. Electrogenic uniport of cations driven by $\Delta \Psi$. Mitochondrial $\text{Ca}^{2+}$ and $\text{K}^+$ uptake belongs to this category.

3. Electroneutral exchange of two metabolites (e.g., $2\text{-oxoglutarate}^{2-}$/malate$^{2-}$). Such transport is driven by concentration gradients of each of the substances transported and as such does not dissipate $\Delta p$.

4. Electrogenic exchange of two metabolites (e.g., $\text{ATP}^{4-}$/ADP$^{3-}$, citrate$^{3-}$/malate$^{2-}$). In this case the direction of exchange is determined by the transmembrane potential. For example, in energized mitochondria the exchange of internal ATP$^{4-}$ against external ADP$^{3-}$ is favored by $\Delta \Psi$ (negative inside), whereas the reverse exchange is hindered. Such a preference disappears in uncoupled mitochondria.

3. PRODUCTION OF REACTIVE OXYGEN SPECIES

The fate of most electrons that enter the respiratory chain is the four-electron reduction of dioxygen ($O_2$) to form water at complex IV. However, “electron leak” from other redox sites in the respiratory chain results in small but significant one-electron reduction of $O_2$ that yields superoxide anion radical $O_2^-$. According to a very rough estimation, about 1% of the total oxygen uptake in mammalian tissues is transformed into this free radical. The superoxide anion, or
its protonated form, $\text{HO}_2^-$ ($pK_a \sim 4.8$), can dismutate to form hydrogen peroxide $\text{H}_2\text{O}_2$ in a reaction catalyzed by superoxide dismutase (SOD):

$$2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$$

(3)

In the presence of transition metal cations, in particular $\text{Fe}^{2+}$ and $\text{Cu}^+$, hydrogen peroxide reacts nonenzymatically in the Fenton reaction, yielding extremely reactive hydroxyl radical $\text{HO}^-$:

$$\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{HO}^- + \text{OH}^- + \text{Fe}^{3+}$$

(4)

The two free radicals, superoxide anion $\text{O}_2^-$ and hydroxyl radical $\text{OH}^-$, along with hydrogen peroxide ($\text{H}_2\text{O}_2$), singlet oxygen ($^1\text{O}_2$) formed in some photochemical reactions, and ozone ($\text{O}_3$), an air pollutant, constitute a class of reactive oxygen species (ROS) that are far more chemically reactive than the “normal” triplet oxygen molecule, $\text{O}_2$. Among them only $\text{O}_2^-$, $\text{H}_2\text{O}_2$, and under specific conditions, $\text{HO}^-$ are physiological metabolites.

It is now generally agreed that the main sites of $\text{O}_2^-$ generation at the level of the mitochondrial electron transport chain are complexes I and III [21,22] (Figure 8). The relative contribution of either complex is not known precisely and may vary among various tissues and depend on metabolic conditions. In complex I the primary source of $\text{O}_2^-$ appears to be one of the iron–sulfur clusters. In complex III the likely mechanism of $\text{O}_2^-$ generation seems to be coenzyme Q cycling, in which ubisemiquinone, a free radical by itself, functions as a redox intermediate on both sides of the inner mitochondrial membrane. Its one-electron autoxidation in the presence of $\text{O}_2$ is likely to generate $\text{O}_2^-$.

A general condition that enables $\text{O}_2^-$ generation is a highly reduced state of the electron carriers at specific sites. Only then may a nonenzymatic “leak” of electrons out of the enzymatic electron transport route (respiratory chain) become possible. This can happen if the respiratory chain is blocked downstream from the particular $\text{O}_2^-$ generation site [e.g., by the microbial product antimycin A (complex III inhibitor) or the plant poison rotenone (complex I inhibitor)]. Another condition is a temporary decrease in oxygen tension (anoxia or hypoxia) followed by reoxygenation. Under physiological conditions, this occurs during reperfusion following ischemia.

In tightly coupled mitochondria, the rate of electron flow through the respiratory chain is limited by the rate of ATP synthesis [i.e., by the availability of ADP (assuming saturating concentration of inorganic phosphate)]. More precisely, it is limited by the rate of proton pumping at the specific coupling sites (i.e., complexes I, III, and IV). In turn, this is limited by the “counterpressure” of the protonmotive force. The rate of electron flow in the respiratory chain is low in the resting state (state 4) and increases greatly in the active state (state 3). Consequently, the reduction status of respiratory chain carriers, especially those of complexes I and III, is higher in the resting state than in the active state. This explains the observation that generally, mitochondrial production of ROS
Figure 8 Sites of ROS generation within the mitochondrial electron transport chain. The superoxide anion (O$_2^−$) is generated mainly at complexes I and III of the respiratory chain and, to a smaller extent, by mitochondrial glycerophosphate dehydrogenase. O$_2^−$ is released into both the matrix side (M side) and the intermembrane (“cytosolic”) side (C side). O$_o^−$ and O$_i^−$ indicate ubisemiquinone radicals at $o$ and $i$ sites of complex III, respectively. Solid lines and arrows show direction of the forward electron transfer; the dotted line indicates the reversed electron transfer driven by the protonmotive force ($\Delta p$). Other abbreviations: Succ, succinate; Fum, fumarate; G3P, sn-glycerol 3-phosphate, DHAP, dihydroxyacetone phosphate; G3P-DH, glycerophosphate dehydrogenase; FMN, flavine mononucleotide; FAD, flavine-adenine dinucleotide; ETF, electron transfer flavoprotein; Q, ubiquinone; FeS, iron-sulfur cluster; R FeS, Rieske iron-sulfur protein; cyt c1, cyt c, cyt a/a3, respective cytochromes. (From Schönfeld, P. and Wojtczak, L. Fatty acids as modulators of the cellular production of reactive oxygen species. Free Radic Biol Med. 2008; 45:231-241; modified.)

is higher in the resting state than in the active state. Therefore, extrapolating to the whole tissue or even to the whole organism, it is incorrect to conclude that ROS generation is proportional to the rate of oxygen consumption. Hence, the value of about 1% reported for the proportion of oxygen consumed being transformed to ROS (see above) should be regarded as an average and as a very rough approximation, especially given high nonphysiological concentrations of O$_2$ ex vivo.

Another factor controlling the rate of ROS generation is the protonmotive force ($\Delta p$). Since its electric component dominates the concentration component (180 to 200 mV for $\Delta \Psi$ compared to 30 to 60 mV for $\Delta \mathrm{pH}$), it can be stated that the second factor regulating the rate of ROS generation is the transmembrane potential. Indeed, a drop of $\Delta \Psi$ by as little as 30 mV that accompanies the transition from state 4 to state 3 can decrease the rate of ROS generation several-fold. Similarly, chemical protonophores such as DNP or CCCP strongly decrease ROS formation in both isolated mitochondria and intact cells and tissues [23]. In many tissues and organs the role of natural regulators of the mitochondrial
protonmotive force is played by the uncoupling proteins (UCPs). Discovered originally in the mammalian thermogenic organ, the brown adipose tissue, and designated as uncoupling protein 1 (UCP1), homologous proteins (i.e., UCP2, UCP3, UCP4) have more recently been found in brain, heart, skeletal muscle, liver, and some other tissues. Although solid experimental evidence is lacking, it is hypothesized that one of the functions of these proteins is to provide subtle control of the mitochondrial protonmotive force, and thus of ROS production [19].

It has to be stressed, however, that Δp affects ROS production primarily by controlling the redox state of respiratory chain components, although some direct effects cannot be excluded. This secondary role is illustrated, for example, by the fact that respiratory inhibitors such as antimycin A and rotenone (in the presence of NAD-linked substrates) increase ROS generation, although they decrease Δp.

A particular case of ROS generation where both redox state and Δp play a decisive role is reversed electron transport. The reversed electron transfer from ubiquinol uphill to complex I is driven by high Δp using electrons derived from the oxidation of succinate to fumarate by complex II or, in some tissues, sn-glycerophosphate dehydrogenase. Due to the reversed electron transfer, in tightly coupled mitochondria, succinate oxidation is able to maintain a higher NADH/NAD⁺ ratio and, consequently, to produce more ROS than in the case of NAD-linked substrates. Both processes are, however, extremely sensitive to Δp, so they can be halted by even a small decrease in the protonmotive force occurring under transition from state 4 to state 3. It seems highly likely that the widely discussed high sensitivity of ROS production to Δp or ΔΨ in intact cells may be due to stopping of the reversed electron transport. The extent of reversed electron transfer, or under what conditions it is a physiological process, remains unresolved.

O₂⁻ generated by the respiratory chain appears on both sides of the inner membrane. It seems likely that the superoxide anion produced at the level of complex I is mostly liberated in the matrix compartment, whereas that produced at complex III may appear on both sides (Figure 8). Apart from the two sites of ROS generation in the respiratory chain, there are a few other enzymes that may produce ROS within the mitochondrion [22,24]. They are 2-oxoglutarate dehydrogenase, one of the tricarboxylic acid cycle enzymes present in the matrix; sn-glycerophosphate dehydrogenase, a flavoprotein enzyme present in some tissues at the external side of the inner membrane; cytochrome b₅ reductase; and monoamine oxidase, both present in the outer mitochondrial membrane. The latter enzyme apparently releases hydrogen peroxide directly rather than superoxide. It oxidizes biogenic amines and is highly active in neurons. In addition, significant amounts of ROS can be produced outside mitochondria: namely, in the endoplasmic reticulum and during some metabolic transformations of polyunsaturated fatty acids. This is, however, outside the scope of the present chapter. It is also important to note that various forms of ROS can be generated within the cell by the action of ionizing and ultraviolet radiation and xenobiotics, including some pharmaceuticals [11] (e.g., the chemotherapeutic agent doxorubicin, or herbicides such as paraquat).
ROS generated during operation of the mitochondrial respiratory chain are generally regarded as by-products of aerobic metabolism. Although they may fulfill some signaling functions, they are mostly harmful to the cell. The hydroxyl radical that originates in the cell from hydrogen peroxide only in the presence of transition metals is extremely reactive and can attack almost any compound in its vicinity. The superoxide anion is more stable but can react with lipids, primarily by attacking double bonds of unsaturated fatty acid moieties, and with proteins and nucleic acids, thus producing wide damage in the cell. Moreover, peroxides of fatty acids can initiate chain reactions that propagate from one acyl chain to another, multiplying the initial damage and destabilizing membranes.

Several systems decompose ROS and thus protect the cell against its noxious actions (see below). However, if the rate of ROS generation increases and/or the protective systems fail, ROS steady-state concentration increases, resulting in oxidative stress. The ultimate effect of such a situation is cell death, either necrotic or programmed (apoptotic).

The protective systems include a number of low-molecular-weight antioxidants and enzymatic systems. The former category includes nutritional products (vitamins) such as ascorbic acid (vitamin C), \( \alpha \)-tocopherol (vitamin E), and \( \beta \)-carotene (provitamin A), as well as intrinsic cellular ingredients, including reduced glutathione (GSH) and reduced pyridine nucleotides NADH and NADPH. It remains unclear to what extent these low-molecular-weight compounds function in the intramitochondrial nonenzymatic defense system. This is in contrast to several enzymatic mechanisms, whose function in combating oxidative stress is well established.

The chain reaction that aims to detoxify the superoxide anion is initiated by superoxide dismutase, as illustrated in reaction (3). In analogy to prokaryotic superoxide dismutase, the mitochondrial enzyme contains manganese atom in its active center (Mn-SOD). This is in contrast to cytosolic superoxide dismutase, which contains zinc and copper atoms (Cu,Zn-SOD). Mn-SOD is located exclusively in the mitochondrial matrix and transforms the \( \mathrm{O}_2^- \) generated therein very efficiently into \( \mathrm{H}_2\mathrm{O}_2 \). This is underscored by the fact that heterozygous Mn-SOD-knockout mice, containing 50% of the normal activity of the enzyme, appear quite normal, yet homozygous animals, essentially lacking mitochondrial SOD, die during the first few weeks after birth [25].

The intermembrane compartment contains Cu,Zn-SOD, which is probably identical or very similar to the cytosolic enzyme. Thus, \( \mathrm{O}_2^- \) generated at the external side of the inner mitochondrial membrane can be transformed efficiently to \( \mathrm{H}_2\mathrm{O}_2 \). It should be noted, however, that the dismutation reaction transforms one reactive oxygen species into another, and although \( \mathrm{H}_2\mathrm{O}_2 \) is not a free radical, it is potentially injurious. The danger presented to cellular integrity and viability by hydrogen peroxide is based on two properties of this compound: (1) it crosses biological membranes readily, in contrast to the limited permeability of the superoxide radical; and (2) it generates extremely reactive hydroxyl radical in the presence of ferrous ions [see reaction (4)]. Therefore, the next step in the protective mechanisms against ROS is removal of hydrogen peroxide by catalase,
a heme enzyme common in various tissues, via the reaction

\[ 2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \]  

(5)

Catalase has diffusion-limited kinetics, and one molecule can turn over millions of molecules of hydrogen peroxide per second. Catalase is located mainly in peroxisomes. Its presence in preparations of isolated mitochondria is due to peroxisomal contamination and can even be regarded as a measure of such contamination. Nevertheless, according to some reports, catalase may be intrinsic to heart mitochondria, making this tissue exceptionally capable of opposing oxidative stress [26].

Enzymes that remove hydrogen peroxide by reducing it with electrons derived from organic compounds are classified as peroxidases. Unspecific peroxidases are common in the cytoplasm of various animal and plant tissues. Intrinsic to mitochondria is glutathione peroxidase, which reacts with reduced glutathione (GSH) as an electron (or oxygen) acceptor, oxidizing it to glutathione disulfide (GSSG), also termed (not quite correctly) oxidized glutathione. Glutathione peroxidase contains selenocysteine in its active center. It is the main, or perhaps the only, enzyme removing \( \text{H}_2\text{O}_2 \) from the mitochondrial matrix, and its effectiveness and efficiency are enabled by a high, millimolar intramitochondrial concentration of GSH.

Another glutathione peroxidase reacts preferentially with phospholipid hydroperoxides, but can also reduce cholesterol peroxides and even \( \text{H}_2\text{O}_2 \) to yield GSSG. This enzyme, phospholipid hydroperoxide glutathione peroxidase, is also a selenoenzyme and is thought to be located inside mitochondria. It can play an important role in repairing biological membranes whose phospholipids have already been peroxidized by various types of ROS. It is abundant in some tissues (e.g., testes) but may be absent in many others.

GSSG resulting from the reactions catalyzed by glutathione peroxidases must be reduced back to GSH to enable the process to continue. This is catalyzed by glutathione reductase, which is present in the mitochondrial matrix, where it utilizes NADPH selectively as the electron donor. In turn, NADPH can be produced by the transhydrogenation reaction

\[ \text{NADH} + \text{NADP}^+ + \Delta p \rightarrow \text{NAD}^+ + \text{NADPH} \]  

(6)

The term \( \Delta p \) on the left side of this reaction indicates that the reaction running from left to right utilizes energy in the form of the protonmotive force. Hence, maintaining a high intramitochondrial concentration of NADPH is connected with energy expenditure. NADPH can also be generated from isocitrate or malate by the action of the respective dehydrogenases, NADP\(^+\)-dependent mitochondrial isocitrate dehydrogenase or, mostly in neurons, decarboxylating malate dehydrogenase (called the malic enzyme). Thus, regeneration of reduced glutathione is an energy-consuming process and can compete with ATP synthesis for the protonmotive force or respiratory substrates, which means that protection against oxidative stress is energetically costly.
There is, however, one ROS-removing process that can, at least theoretically, provide energy to mitochondria instead of utilizing it. This is the oxidation of $\text{O}_2^-$ by cytochrome $c$ present in the intermembrane compartment. Since cytochrome $c$ is bound loosely to the outer surface of the inner mitochondrial membrane, it is also present at low, submillimolar concentration in the free form between the inner and the outer membranes. This fraction of free cytochrome $c$ can react nonenzymatically in a one-electron process with the superoxide radical according to the reaction

$$\text{O}_2^- + \text{cyt. } c(\text{Fe}^{3+}) \rightarrow \text{O}_2 + \text{cyt. } c(\text{Fe}^{2+})$$

(7)

Reduced cytochrome $c$ can subsequently be reoxidized by complex IV of the inner mitochondrial membrane, thus providing electrons to the final step of the respiratory chain that is coupled to proton pumping and $\Delta \text{p}$ formation [27].

Under normal conditions, all these ROS-metabolizing processes are sufficient to keep intramitochondrial steady-state concentrations of $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ at physiological submicromolar levels. Moreover, it is hypothesized that mitochondria can function as a sink for ROS produced extramitochondrially [24]. Oxidative pathology emerges only after failure of one or more of these scavenging systems and/or substantial elevation of ROS generation, conditions generally termed oxidative stress.

The evolutionary adaptations to prevent the noxious effects of oxygen free radicals are the price that organisms living in oxygen-rich environment have to pay for highly efficient aerobic ATP synthesis. Chronic exposure of mitochondria to relatively high ROS concentrations increases the probability of mtDNA mutations, especially because mtDNA is not protected by histones and contains no introns and is therefore more susceptible than nuclear DNA to oxidative damage. Gradual damage of mtDNA during the human life span results in a progressive decrease in the efficiency of OXPHOS. This in turn may promote accelerated ROS formation, which further enhances mtDNA mutations. Such a vicious cycle is an unavoidable consequence of aerobic poise and supports the mitochondrial theory of aging [28–32]. Gradual loss of ATP generating capacity undermines many crucial cellular processes and has been implicated in many degenerative diseases. In addition, some pathologies, which may lead to mitochondrial stress, such as inflammatory diseases, excessive physical exercise, and ischemic insult followed by reperfusion, can enhance ROS generation and therefore may increase the probability of mtDNA mutation above normal levels.

4. CALCIUM SIGNALING

Mitochondrial $\text{Ca}^{2+}$ uptake, extrusion, and accumulation are key to cellular calcium homeostasis. As discussed above, $\text{Ca}^{2+}$ influx through the inner mitochondrial membrane is driven by $\Delta \Psi$; hence, it is sensitive to factors that may affect the mitochondrial energy state, such as uncouplers and respiratory chain
inhibitors. The bulk cytosolic Ca$^{2+}$ concentration in the resting cell is about 100 nM, so that given a $\Delta \Psi$ value of 180 mV (negative inside), mitochondrial Ca$^{2+}$ concentration at equilibrium should theoretically reach the value of 100 mM (one order of magnitude for each 30 mV of $\Delta \Psi$). In fact, this does not occur since Ca$^{2+}$ influx is effectively counterbalanced by Ca$^{2+}$ efflux, which occurs via an electrogenic Ca$^{2+}$/3Na$^+$ antiporter or, in some cells with low sodium clearance (e.g., hepatocytes), as electroneutral Ca$^{2+}$/2H$^+$ exchange. Sodium entering the mitochondrial matrix is removed by the Na$^+$/H$^+$ exchanger. Thus, the net balance between Ca$^{2+}$ entry and release is maintained at the expense of the proton circuit [33].

The mitochondrial Ca$^{2+}$ uniporter, through which cytosolic calcium enters the matrix, displays a low affinity toward Ca$^{2+}$, with a $K_d$ value greater than 10 µM in the presence of physiological Mg$^{2+}$ concentrations. This, together with a high activity of the Ca$^{2+}$/3Na$^+$ antiporter, makes mitochondrial Ca$^{2+}$ accumulation inefficient until cytosolic Ca$^{2+}$ concentration reaches a threshold of about 400 nM. Therefore, mitochondria should not be considered as Ca$^{2+}$ storage organelles that accumulate calcium in resting cells. In stimulated cells, bulk cytosolic Ca$^{2+}$ concentration increases up to 1 µM, but locally, in close proximity to Ca$^{2+}$ channels in the endoplasmic reticulum (ER) and in the plasma membrane, it may reach much higher values. Mitochondria located in such subcompartments accumulate Ca$^{2+}$ efficiently and decrease the local Ca$^{2+}$ concentration. Such Ca$^{2+}$-buffering activity of mitochondria may affect many calcium-dependent processes, including Ca$^{2+}$ entry through calcium channels and its removal by Ca$^{2+}$-ATPases [34,35]. Therefore, mitochondria modulate the intensity of intracellular calcium signals originating from both ER and the extracellular space [36]. In this way, mitochondria can also control calcium waves and oscillations spreading throughout excited cells [37,38].

Matrix Ca$^{2+}$ is chelated by proteins, nucleotides, and phosphate, and is released readily when cytosolic Ca$^{2+}$ returns to lower resting levels. It must be emphasized that a slight increase in the mitochondrial Ca$^{2+}$ concentration stimulates energy metabolism, as calcium activates pyruvate dehydrogenase and two dehydrogenases of the tricarboxylic acid cycle. On the other hand, excessive mitochondrial Ca$^{2+}$ accumulation fosters activation of the permeability transition pore (PTP) and therefore may be harmful to mitochondria and eventually to the cell [29,39] (see Section 5). However, the PTP can also operate in the low-conductance mode that allows mitochondria to release excess calcium, thus reducing the risk of mitochondrial damage without dissipating $\Delta \psi$ completely [40]. In such a case, the PTP prevents mitochondrial malfunction and supports cell survival. Moreover, a limited opening of the PTP slightly decreases $\Delta \psi$, which in turn reduces mitochondrial ROS formation.

Intracellular organization of the mitochondrial network, which bridges the sub-plasma membrane space and ER, gives a structural basis for intramitochondrial Ca$^{2+}$ transfer from the plasma membrane calcium channels to Ca$^{2+}$-ATPases that pump Ca$^{2+}$ into intracellular stores [41]. Apart from calcium channels and
pumps located in the plasma membrane and in the endoplasmic reticulum, respectively, this phenomenon involves mitochondrial Ca$^{2+}$ uniporters and mitochondrial Ca$^{2+}$/3Na$^+$ antiporters. Calcium released from mitochondria into a limited space makes a local “hot spot” in the proximity of Ca$^{2+}$-ATPase. It increases the rate of filling up the calcium stores and allows for their reloading without an excessive increase in the cytosolic Ca$^{2+}$ concentration. On the other hand, mitochondria located very close to Ca$^{2+}$ channels in ER membranes (coupled to IP$_3$-dependent or ryanodin receptors) may sense local increases in the cytosolic Ca$^{2+}$ concentration in very discrete junctions between ER and mitochondria, so they may take up Ca$^{2+}$ almost directly from the intracellular stores [41].

Perturbations in the mitochondrial energy metabolism affecting /$\Delta$$/Psi$/ interfere with cellular calcium homeostasis and may result in serious consequences for the cell. The decrease in oxidative phosphorylation and hence the resulting ATP deficiency limit the rate of Ca$^{2+}$ removal from the cytosol to the extracellular space as well as Ca$^{2+}$ sequestration in intracellular calcium stores. This leads to a harmful overactivation of numerous calcium-dependent enzymes, such as calpains, phospholipases A$_2$, and protein kinases C. Moreover, prolonged increases in the poststimulatory cytosolic Ca$^{2+}$ concentration decrease the excitability of electrically excitable cells. In the case of neurons, it delays recovery of the rest potential that may affect brain plasticity. Such phenomenon is attributed to age-related ROS-induced impairment of OXPHOS [42]. On the other hand, the reduced Ca$^{2+}$-buffering capacity of mitochondria strongly affects cellular calcium signaling because of the lowered ability of mitochondria to regulate local and global calcium events such as spikes, sparks, waves, and oscillations [37,43].

5. MITOCHONDRIA AND CELL DEATH

It is paradoxical that mitochondria, which are indispensable for cell survival, are also necessary for cell suicidal death. This programmed cell death, also called apoptosis, is a complex sequence of events aimed to eliminate single cells or their assemblies when their natural biological function has come to an end or when a cell has become damaged or mutated to such an extent that its further existence might be deleterious to the whole organism. In particular, apoptosis occurs in embryogenesis, metamorphosis, and in the growth and maturation of individual organs. Apoptosis is also believed to eliminate cells whose metabolism and genomic organization have undergone transformations that may lead to malignancy. Thus, apoptosis is one of the main natural mechanisms protecting against cancer development. On the other hand, the increased propensity of a cell to undergo apoptotic decay may give rise to a series of pathologies, such as neurodegenerative diseases and tissue damage, that develop as a consequence of ischemia, in particular in heart and brain.

In general, apoptosis may proceed by two partially interdependent routes, the death receptor pathway and the mitochondrial pathway [8,44,45]. The former is initiated by ligation of death receptors at the cell surface, whereas the latter
originates in mitochondria. In this case, one of the early events is the release of cytochrome c and some other peptides from mitochondria into the cytosolic compartment. The mechanism by which cytochrome c is liberated from mitochondria to the cytosol is still debated. Earlier hypotheses assumed that mitochondrial swelling causes disruption of the outer membrane. More recent reports indicate, however, that cytochrome c is also released under conditions where the outer membrane retains its integrity. A decisive role in this process is played by the mitochondrial permeability transition pore and proapoptotic proteins of the Bcl-2 family: in particular, Bid and Bax.

One of the factors that can initiate this process is oxidative stress and the resulting oxidative attack of reactive oxygen species on phospholipid components of the inner mitochondrial membrane, particularly cardiolipin. Cytochrome c is normally bound to the inner membrane by electrostatic interactions with negatively charged cardiolipin. As cardiolipin is rich in polyunsaturated fatty acids, mostly linolenic acid, it easily undergoes peroxidation, which changes its physicochemical properties drastically and may lead to a partial desorption of bound cytochrome c. As a result, the concentration of free cytochrome c in the intermembrane compartment, normally at low submillimolar levels, may increase sharply, promoting leakage into the cytosol [46] (Figure 9).

Apoptosis has often been observed to be accompanied by mitochondrial fission [48,49]. It remains, however, debatable whether this change in the structure of the mitochondrial network is related to the liberation of cytochrome c and other proapoptotic factors from mitochondria [50].

Cytochrome c released to the cytosol participates in the formation of a multiprotein complex called apoptosome. Together with other components of this complex, and in the presence of dATP or ATP, the apoptosome activates caspase-9. This is a representative of a large class of cysteine proteases that cleave their substrates after the aspartic acid moiety (hence the term caspases). Activation of caspase-9 is, by itself, an autocatalytic proteolysis that transforms procaspase-9 into its active form. Caspase-9 belongs to a class of initiator caspases, as it activates a series of other caspases, called effector caspases, in particular caspase-3 and caspase-7. Activated caspases are mainly responsible for degradation of the cell that is characteristic of the terminal phase of apoptosis. However, accidental activation of one of the initiator caspases might also trigger a chain of reactions eventually leading to cell destruction. To avoid such an inadvertent course of events, cells also contain a protective system in which the central role is played by a family of caspase-inhibitor proteins, IAPs (inhibitors of apoptosis proteins). Thus, to enable programmed cell death to proceed, IAPs are removed or otherwise neutralized concomitant with activation of the caspases. This function is fulfilled by another protein, Smac (second mitochondrial activator of caspases; also called Diablo), that is released from the mitochondrial intermembrane space together with cytochrome c and other proapoptotic proteins.

As mentioned above, the PTP seems crucial for the release of proapoptotic factors. This pore is located in the contact sites between the outer and inner mitochondrial membranes and in its open state enables free passage of
Figure 9  Schematic representation of mechanisms accounting for outer mitochondrial membrane permeabilization and the release of cytochrome c. (A) Induction of permeability transition pore opening, leading to matrix expansion and rupture of the outer membrane. (B) Bax-mediated permeabilization of the outer mitochondrial membrane, involving tBid-induced Bax insertion and homooligomerization that can be inhibited by Bcl-2 or Bcl-XL. (C) Peroxidation of cardiolipin is a key first step in mobilizing cytochrome c from the inner mitochondrial membrane prior to Bax-induced (b) permeabilization of the outer membrane. (From Robertson et al. [47] with permission of Macmillan Publishers Ltd., copyright © 2003.)

low-molecular-weight compounds, up to 1.5 kDa, between the mitochondrial inner compartment (matrix) and the cytosol [51,52]. It is formed by a complex assembly of several proteins originating from the outer mitochondrial membrane (porin), the inner membrane (adenine nucleotide translocase), and the matrix (cyclophilin D) (Figure 10). Opening of the PTP is favored by factors such as $Ca^{2+}$ accumulation in mitochondria, reactive oxygen species, and low $\Delta\Psi$. The PTP is believed to be a “safety valve” against calcium overload of the mitochondrial inner compartment. Its flickering may also be one of the factors responsible for a limited “proton leak” through the inner membrane of coupled mitochondria. PTP opening results in large-scale mitochondrial swelling. Such swelling, leading to rupture of the outer mitochondrial membrane and liberation of soluble proteins from the intermembrane compartment to the extramitochondrial space, was initially believed to be one of the underlying factors of apoptosis. Subsequent research revealed, however, that it may not be so, because large-scale
swelling, which is due to a difference in the colloidal osmotic pressure inside the matrix compartment and the external medium, is much less pronounced in mitochondria within the cell than in isolated mitochondria suspended in isotonic saline or sucrose media. Also, multiple ultrastructural observations do not show a link between mitochondrial swelling and rupture of the outer membrane with the onset and the progress of apoptosis.

The PTP alone is too narrow to allow passage of cytochrome c (13 kDa), apoptosis-inducing factor (AIF), and other proapoptotic proteins. Moreover, the pore connects the cytosolic compartment with the matrix compartment, not the intermembrane space where the aforementioned apoptosis-inducing proteins are located. Therefore, although a relation between PTP opening and the onset of apoptosis has been well documented, connections between these events are complex. It has been shown, however, that a channel permeable to cytochrome c, AIF, and other proteins of the intermembrane space is formed by association of the PTP with the proapoptotic proteins Bax and Bak. In nonapoptotic cells, these two proteins are located in the cytosol or are loosely bound to the outer mitochondrial membrane in monomeric forms. Upon a death stimulus, another

Figure 10 Model of the contact site between the outer and inner mitochondrial membranes that may function as the permeability transition pore. Indications: VDAC, voltage-dependent anion channel (mitochondrial porin); ANT, adenine nucleotide translocase; Cyp D, cyclophilin D; HK, hexokinase; PBR, peripheral benzodiazepine receptor; Bcl-2, antiapoptotic protein Bcl-2. Cytochrome c molecules associated partially with the outer face of the inner membrane and partially free in the intermembrane space are indicated by red circles. (Drawing by M. R. Wieckowski.) (See insert for color representation of figure.)
proapoptotic protein, Bid, undergoes a proteolytic cleavage, and its C-terminal truncated derivative, t-Bid, induces homooligomerization of Bax and Bak, which then associate more firmly to the outer membrane, making it permeable to cytochrome c. This association and its pore-forming activity are prevented by the antiapoptotic proteins Bcl-2 and Bcl-XL. Thus, a subtle balance between these proapoptotic and antiapoptotic proteins and their interactions with the PTP are decisive for the survival or the apoptotic death of the cell. This balance can be affected by a number of mitochondria-targeted drugs. To make the process even more complex, it has been observed that some heat-shock proteins, in particular HSP70, may also prevent cytochrome c release or can somehow “neutralize” cytochrome c that has already been released.

Mitochondria also release endonuclease G, which is involved in DNA degradation. Some other nucleases become activated by caspases. These nucleases are decisive in internucleosomal cleavage of DNA in cells undergoing apoptosis.

As mentioned above, one of the apoptosis-promoting factors is reactive oxygen species [53]. Excessive production of ROS in the cell can be induced by a number of xenobiotics, transition metal ions, and ultraviolet and ionizing radiations. ROS action on mitochondria results in both a detachment of cytochrome c from the inner membrane and opening of the PTP, thus promoting liberation of cytochrome c to the cytosol.

Ionizing radiation (x-ray and γ radiation), often used in cancer therapy, also acts by inducing apoptosis. Being more energetic than ultraviolet radiation, it also affects DNA and thus initiates both DNA- and mitochondrial-linked apoptosis pathways. Similarly, several anticancer drugs exert their therapeutic effect by inducing the apoptosis of malignant cells. In general, they act by inducing intracellular ROS production (e.g., doxorubicin). The increased level of ROS not only promotes PTP opening but, as mentioned above, also results in the peroxidation of polyunsaturated fatty acid moieties in the phospholipid bilayer of the inner membrane, in particular of cardiolipin, thus promoting desorption of cytochrome c. It is often stressed that massive release of cytochrome c from mitochondria requires not only permeabilization of the outer membrane but also an increased level of free, unbound cytochrome c in the intermembrane compartment. A simplified scheme of mitochondrial events leading to apoptosis is shown in Figure 11.

In contrast to apoptosis, which can be regarded as a controlled process, necrosis is defined as an uncontrolled cell death leading to nonselective cell damage. It usually results from major cell injury and disruption of vital cell functions such as energy production and selective permeability of cell membranes. Necrosis is a pathological rather than a physiological process and is usually followed by inflammatory reactions of adjacent cells and tissues. Similar to apoptosis, necrosis can also be induced by extracellular pathological disturbances such as ischemia, trauma, and some neurodegenerative disorders. The most characteristic features of cells dying a necrotic death are mitochondrial permeabilization, disruption of lysosomes, and loss of osmotic balance between intra- and extracellular fluids. This latter event results in an increase in cell volume, eventually leading to plasma...
Figure 11  Mitochondrial pathway of apoptosis. The pathway is triggered by various “death signals”, as reactive oxygen species (ROS), DNA damage, and so on, that promote binding of the proapoptotic protein Bax with the outer mitochondrial membrane, probably at the contact sites between the two membranes, and its association with the permeability transition pore (PTP). This enables the release of cytochrome c (circles) and other proapoptotic proteins (the apoptosis-inducing factor AIF, endonuclease G, Smac, etc; squares) from the intermembrane compartment to the cytosol. An elevated intramitochondrial Ca$^{2+}$ level and ROS production facilitate this process by promoting PTP opening. Once in the cytosol, cytochrome c, in cooperation with a cytosolic factor, Apaf-1 (not indicated), activates caspase-9 and subsequently other members of the caspase family, thus initiating self-digestion of the cell and nuclear DNA fragmentation, eventually leading to apoptotic cell death. Association of Bax with mitochondria is prevented by the antiapoptotic protein Bcl-2. ROS can be decomposed by Mn-containing (mitochondrial) and Cu,Zn-containing (cytosolic) superoxide dismutases (SOD), catalase, and glutathione peroxidase (GPx). Stimulation of ROS production is exemplified here by ultraviolet and ionizing radiation and by two anticancer drugs, adriamycin and BMD188 [cis-1-hydroxy-4-(1-naphthyl)-6-octylpiperidine-2-one]. Activation is indicated by an encircled plus sign, and inhibition by an encircled minus sign. (Modified from Szewczyk and Wojtczak [11], with permission of the publisher.)
membrane rupture and leakage of the intracellular content (ions, metabolites, and proteins).

One of the major biochemical parameters determining the fate of cells challenged by life-threatening stimuli is their energy level. Whereas apoptosis needs a certain ATP content, necrotic cell death does not require energy. Presumably, a switch between apoptotic and necrotic cell death depends on the mitochondrial energy state and the extent of mitochondrial impairment. For example, it has been shown [54,55] that continuous ATP supply in hepatocytes challenged by ischemia or reperfusion stress can switch the cells from the necrotic to the apoptotic mode of dying. This confirms that cell depletion of ATP is a commitment step for necrosis.

Apart from apoptosis and necrosis, autophagy is another mode of elimination of unwanted cells [56]. Autophagy enables the removal of long-lived proteins and damaged organelles inside intracellular digesting vesicles. This mechanism offers recycling and salvage of intracellular material as well as delivering essential components during temporary interruption of nutrient supply. In this sense, autophagy is a pro-survival process. On the other hand, autophagy may act as a death mechanism, especially when apoptotic cell death is prevented [57]. Cellular autophagy-related signaling pathways have been studied intensely primarily in yeast, but their mechanisms have not yet been fully clarified.

Autophagy is also considered as a mechanism of removal of damaged organelles or those rendered unnecessary because of changing environmental or nutritional conditions [58]. It seems that mitochondrial turnover, which allows replacing of aged or impaired mitochondria, is based on their degradation in intracellular digestive vesicles, autophagosomes. It has been found, at least in yeast, that mitochondrial autophagy needs participation of the outer membrane Uth1p protein [59]. This points to a selective process. Mammalian analogs of Uth1p protein have not yet been found. It is suggested that apart from its role in apoptotic and necrotic cell death, mitochondrial permeability transition may also stimulate autophagy to remove damaged mitochondria in intact cells. This mechanism may protect the whole cell against apoptotic or necrotic death by decreasing the proportion of damaged mitochondria that display extensive ROS production, Ca2+ overload, and activation of the mitochondrial permeability transition pore. Thus, the PTP seems to contribute not only to cell death but may also trigger selective elimination of those mitochondria that may expose the cell to enhanced risk of apoptosis or necrosis [60].

6. CONCLUDING REMARKS: MITOCHONDRIA AS A PHARMACOLOGICAL TARGET

The aim of this overview was to introduce multiple aspects of mitochondrial biology with particular attention to the roles these organelles play in cell survival and cell death. The importance of mitochondria in providing the cell with the energy required to maintain integrity and viability, and the mechanisms whereby
this is accomplished were discussed, as was the mitochondrial contributions to suicidal cell death, a process of vital importance for all multicellular organisms.

Their predominant roles as ATP suppliers, as cellular ROS producers, and as important regulators of apoptosis, renders mitochondria promising targets for pharmacological interventions [11]. Currently, most efforts along these lines focus on preventing mitochondrial and cellular oxidative damage that arise from multiple pathological conditions, such as ischemia and reperfusion-evoked cell damage, diabetes, and neurodegenerative diseases. Examples of such possible pharmacological interventions are (i) induction of mitochondria-dependent apoptosis with prospective importance for cancer therapy, (ii) controlling of mitochondrial permeability transition pore as a possible means for prevention of ischemia and reperfusion-related cell injury, (iii) decreasing mitochondrial membrane potential to increase oxidation of intracellular lipid deposits and reduce ROS production in treatments of obesity and diabetes [61], and (iv) moderate inhibition of the respiratory chain to limit ATP availability for hepatic gluconeogenesis in diabetes [62]. In addition, mitochondria are prospective targets for gene therapy in case of diseases caused by mutations in the mitochondrial genome [63].

Systemic administration of drugs selectively targeting mitochondrial functions presents a number of problems. Therefore, much attention has been paid to mitochondrially-targeted drugs, which may reach these organelles without affecting other intracellular structures and extramitochondrial processes. Such selective drug delivery may be accomplished by using specific carries that can bind to and enter mitochondria. Among them are delocalized lipophilic cations, which accumulate in the mitochondrial matrix or within the inner mitochondrial membrane at the expense of mitochondrial ΔΨ [64]. Other examples are small peptides that selectively partition to the inner mitochondrial membrane [65], liposomes consisting of self-assembling mitoondriotropic compounds [66], and chimeras composed of mitochondrial signalling peptides combined with other proteins or DNA [67]. Such selective mitochondria-targeted drug delivery seems to be the most promising approach to prevent or treat mitochondrial diseases. However, because of unresolved questions concerning drug delivery to appropriate organs and possible side effects of molecules used as drug carriers, these techniques are still at the stage of experimentation. Many of these issues will be discussed further in the ensuing chapters.

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