Chapter 6
Energy Conservation in Metabolism: The Mechanisms of ATP Synthesis

In living organisms, different types of energy are always interconverting into one another within the cell to allow the distinct cellular functions to be performed. This can be illustrated by several examples, such as the conversion of the energy of light into chemical energy in photosynthetic organisms or the chemical energy into mechanical energy in muscle contraction. Also, virtually all the cells need to use chemical energy to transport ions and other compounds across a membrane, generating a concentration gradient and thus converting chemical into osmotic energy. Finally, chemical energy is continuously converted in other forms of chemical energy during the biosynthesis of new molecules in cellular metabolism.

In the beginning of the twentieth century, a set of experiments carried out by Arthur Harden and William J. Young, in which they showed that phosphate is essential for yeast alcoholic fermentation, started a new era for the understanding on how energy is obtained from the environment and stored within the cells for later use. This discovery was the first association between phosphate and energy transformations in living cells, paving the way for the subsequent identification of ATP, more specifically its phosphoanhydride bond, as the main cellular energy carrier.

In this chapter, we will discuss the principles and the most relevant steps of the main processes of ATP synthesis in heterotrophic cells.

Heterotrophic organisms conserve the energy of nutrient molecules by coupling the breaking of their chemical bonds to the synthesis of ATP, which occurs through two distinct mechanisms.

The first mechanism of ATP synthesis to be identified is known as substrate-level phosphorylation. It does not depend on oxygen and thus may occur in anaerobiosis. The general principle involved in ATP synthesis through this mechanism is the formation of a phosphorylated molecule that presents a high-energy phosphate bond or, in a more precise term coined by Fritz Lipmann, a high potential of transferring its phosphoryl group, which is used to phosphorylate ADP, generating ATP.

The second mechanism of ATP synthesis is known as oxidative phosphorylation. It depends on organized membranes and, in eukaryotic cells, takes place in mitochondria. The unraveling of this mechanism was possible due to someone who

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could see beyond the energy of the chemical bonds. This man was Peter Mitchell, who postulated the revolutionary chemiosmotic theory, which states that the synthesis of ATP from ADP and inorganic phosphate (Pi) is driven by the pH gradient across the mitochondria inner membrane and the consequent formation of a transmembrane electrical potential.

6.1 Fermentation: The Anaerobic Pathway for ATP Synthesis

As the first forms of life appeared on Earth when the atmosphere contained no oxygen yet, the anaerobic use of glucose may be considered the most ancient metabolic pathway of energy conservation.

In humans, this pathway is still very important since there are cell types that lack the oxidative apparatus, specially the mitochondria, organelles in which the oxidative phosphorylation takes place. These cells include the mature erythrocytes, cells from the crystalline, and some cells of the retina. There are also many situations in which oxygen availability is limited, and thus the ATP synthesis through fermentation becomes essential. This is the case of muscle cells in intense contraction activity, working in low oxygen conditions due to the adrenaline-induced contraction of peripheral blood vessels (see Chap. 10). There are also pathological situations in which fermentation overcomes oxidative metabolism even in the presence of oxygen. This is the case of cancer cells, in what is called the "Warburg effect" (see Box 6.4).

It is also important to note that only sugars can be used as energy source in anaerobic conditions. The degradation of the other nutrient molecules, the lipids and the proteins, depends on metabolic pathways that occur in mitochondria and

culminate with ATP synthesis through oxidative phosphorylation, which requires oxygen as the final substrate (see details in Sect. 6.2).

6.1.1 A Historical Perspective of the Discovery of the Fermentation Process

Since ancient times, man has dealt with processes involving fermentation, although only recently fermentation has been understood as a metabolic pathway associated to the reactions of energy transformation essential for life.

Fermentation is the basis of the production of bread, beer, and wine, activities that accompanied humans since the early civilizations. The term fermentation arose from these practices, coming from the Roman word *fermentare*, which is related to the formation of bubbles. Thus, fermentation was firstly associated to a process in which a gas was produced. Although now we know that depending on the organism fermentation leads to different end products, which do not always include a gas, in the alcoholic fermentation, which occurs during the production of bread, beer, and wine, sugars from fruits or cereals are converted into ethanol and CO_2, which is the gas released.

Despite several advances in the scientific studies on fermentation during the nineteenth century, Louis Pasteur was the first to connect living organisms to fermentation, which was the basis for its understanding as a biological process (see Box 6.1).

Box 6.1: The Impact of Pasteur's Ideas About Fermentation

The concept that fermentation was performed by living organisms was not readily accepted when proposed by Pasteur, especially because it seemed to reinforce the theory of vitalism, which assumed that organic substances in living organisms could be formed only under the influence of a mysterious "vital force." Jakob Berzelius, Justus von Liebig, and Friedrich Wohler, important chemists from that time, were strongly against the vitalistic ideas, and although they were right in their concepts on the chemical processes, their convictions made them very reluctant to acknowledge the valuable contributions of Pasteur's observations. This can be illustrated by the ironic text published by Woehler and von Liebig in 1839 in the Annals of Chemistry regarding the participation of yeast in the transformation of sugars in ethanol and CO_2 during beer production: "Beer yeast, when dispersed in water, breaks down into an infinite number of small spheres. If these spheres are transferred into an aqueous solution of sugar, they develop into small animals. They are endowed with a sort of suction trunk with which they gulp the sugar from the solution. Digestion is immediate and clearly recognizable because of the discharge of excrements. These animals evacuate ethyl alcohol from their bowels and carbon dioxide from their urinary organs. Thus one can observe how a specifically lighter fluid is extruded from the anus and rises vertically, whereas a stream of carbon dioxide is ejected at very short intervals from their enormously large genitals."

Pasteur observed microscopic globules in a wine sample (Fig. 6.1a) and sustained that these globules were living microorganisms responsible for the sugar transformation into ethanol and CO_2. He also demonstrated that each type of fermentation is linked to a specific microorganism or, as he named, a specific "ferment." Interestingly, similar globules have been described in a sample of beer very earlier, in 1680, by Anton van Leeuwenhoek, the pioneer of the use of the microscope (Fig. 6.1b), although there is no evidence that van Leeuwenhoek had associated these globules to living organisms.

Besides associating fermentation to the presence of living organisms, Pasteur also carried out crucial experiments showing that it occurred in the absence of oxygen. These experiments discredit the current view of fermentation as a chemical process resulted from the reaction between oxygen and sugars, reinforcing the idea that fermentation was a biological process. Pasteur's findings also allowed the formulation of other two important biological concepts derived from his experiments. The first one was that some forms of life can exist without oxygen, in what he referred in one of his most famous articles, published in 1861, as *la vie sans l´air* (the life without air). The second concept came from an intriguing observation that he made in those experiments: more CO_2 was produced by yeast in the absence than in the presence of oxygen, a phenomenon known as the "Pasteur effect" (Fig. 6.1c), which is now explained by the fact that the number of ATP molecules synthesized per glucose molecule in fermentation is much lower than that in oxidative phosphorylation (see in Chap. 4 the stoichiometry of ATP synthesis in the oxidative metabolism of different nutrients).

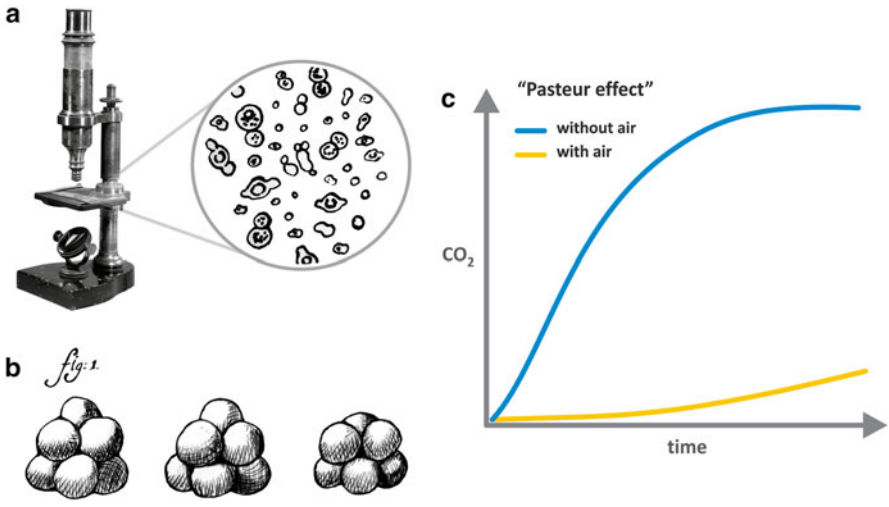


Fig. 6.1 (**a**) The microscope used by Pasteur to observe the wine samples and his drawing representing the "globules" he had observed in the sample (Adapted from Pasteur's book "Études sur la bière," 1876). (**b**) Drawing by van Leeuwenhoek, reporting in 1680 the observation of a beer sample in the microscope that he developed. (**c**) Representation of the "Pasteur effect," in which the production of CO_2 by yeast is much higher in the absence than in the presence of oxygen

The next significant advance in the understanding of fermentation came from an unexpected observation. Eduard Buchner, working with his brother Hans in the preparation of a yeast extract to treat patients with tuberculosis, discovered that even when all the yeast cells were completely disrupted, fermentation continued to proceed normally. This finding marks the end of the vitalism theory by showing that the presence of living cells was not necessary for fermentation and can be regarded as the dawn of biochemistry, introducing the concept that biological reactions are catalyzed by molecules, which Buchner called zymases, the term firstly used to refer to what we now call enzymes. Due to the great impact of his discoveries, Eduard Buchner was the first scientist to be awarded the Nobel Prize in Chemistry, in 1907.

Finally, we must comment the experiment by Harden and Young, mentioned in the beginning of this chapter. Using the cell-free system developed by the Buchners, they observed that the fermentation rate, which decreased with time, could be restored with the addition of salts of phosphoric acid (Fig. 6.2). This was the first evidence that phosphate was involved in cell metabolism, opening the way in the subsequent years to the recognition of the ubiquitous and the crucially important biological phosphorylations in life.

Few years later, Otto Meyerhof, studying muscle contraction in different organisms, showed that contraction occurred in the absence of oxygen, a similar phenomenon to that observed by Pasteur for yeast alcoholic fermentation (Fig. 6.3a). However, in the case of muscle cell fermentation, the end product was lactate. The molecular similarity of the end products of alcoholic and lactic fermentations suggested that both processes could be equivalent (Fig. 6.3b).

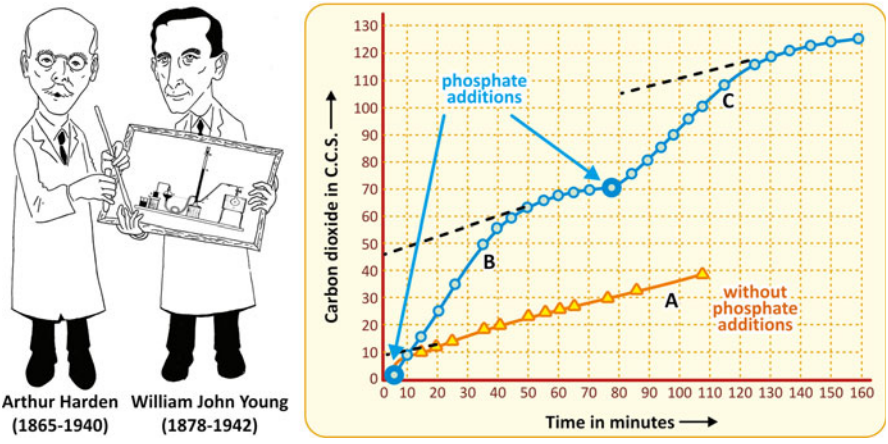


Fig. 6.2 Original figure from the article by Harden and Young published in 1906 (Adapted from Harden & Young. Proc. Royal Soc. Lond. B 77:405–520, 1906), showing the production of CO_2 by yeast fermenting glucose in the absence of any addition (curve A) and when salts of phosphoric acid were added at the indicated times (curves B and C)

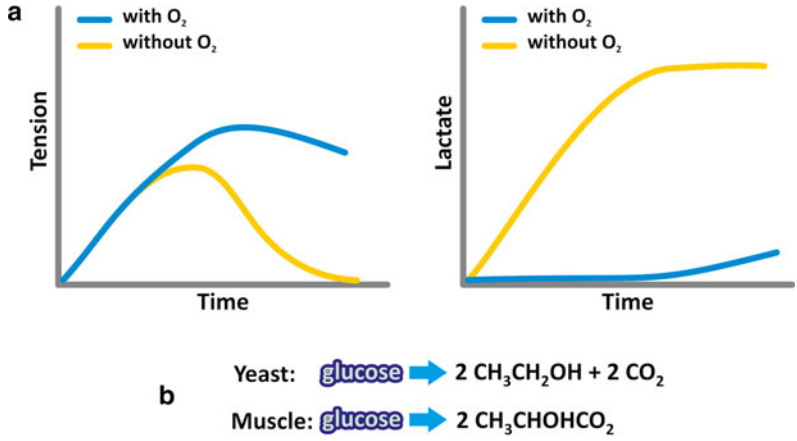


Fig. 6.3 (**a**) Schematic representation of the results obtained by Otto Meyerhof when a muscle fiber was incubated with glucose; tension due to contraction and lactate production were measured as a function of time. (**b**) The analysis of the end products of both, alcoholic fermentation performed by yeast and lactic fermentation performed by the muscular fibers, shows great similarity

Additionally, the same requirement of phosphate was observed for fermentation in the muscle cells. Now we know that indeed the processes are identical, except for the last reaction (see next section).

6.1.2 An Overview of the ATP Synthesis by Substrate-Level Phosphorylation During Fermentation

The general mechanism of ATP synthesis that occurs during fermentation consists in a series of reactions that rearranges the molecular structure of an initially phosphorylated monosaccharide, which is a hexose phosphate, in such a way as to form phosphorylated compounds with high potential of transferring their phosphoryl group (see Box 6.2). These high-energy intermediates are triose-phosphate molecules originated from a cleavage step and whose phosphoryl group is transferred to ADP, generating ATP (Fig. 6.4a). This process of ATP synthesis is named substrate-level phosphorylation, since the phosphoryl group of an intermediate of the pathway is directly used to phosphorylate ADP.

The initial substrate for fermentation is usually glucose, the most abundant monosaccharide derived from a regular human diet. The two initial steps of phosphorylation use ATP as the phosphoryl group donor (Fig. 6.4a). This may seem nonsensical at a first glance, if we think that this is a metabolic pathway for the synthesis of ATP. However, as it will become clear through the analysis of the stoichiometry of the entire pathway, although two ATP molecules are used for each

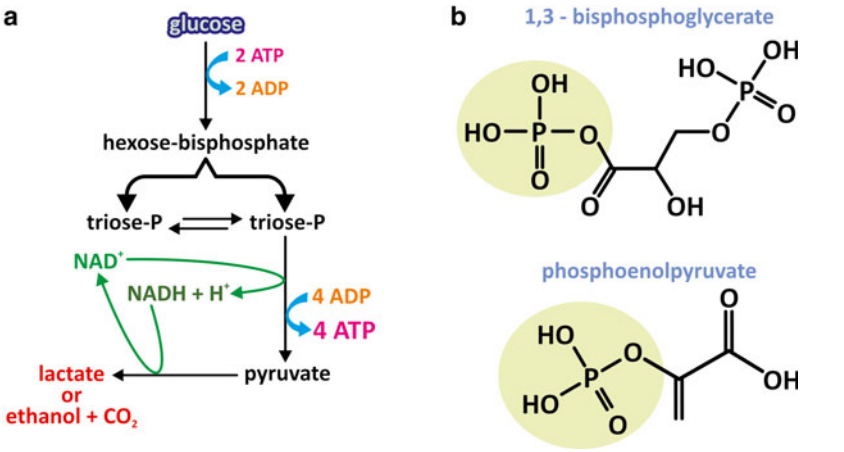


Fig. 6.4 General overview of the essentials of the fermentation process. (**a**) The pathway initiates with a hexose (usually glucose). After two steps of phosphorylation with ATP as the phosphate donor, the resultant hexose bisphosphate is cleaved into two triose phosphates, which may interconvert. One of them follows the pathway, which includes one NAD^+-dependent oxidation reaction and two transfers of the phosphate group of a high-energy triose phosphate to ADP, generating ATP and, at the second of those reactions, pyruvate. Pyruvate must be reduced to allow NADH reoxidation. The main end products of fermentation are lactate or ethanol and CO_2, depending on the organism. (**b**) Chemical structures of the high-energy triose-phosphate molecules 1,3-bisphosphoglycerate and phosphoenolpyruvate

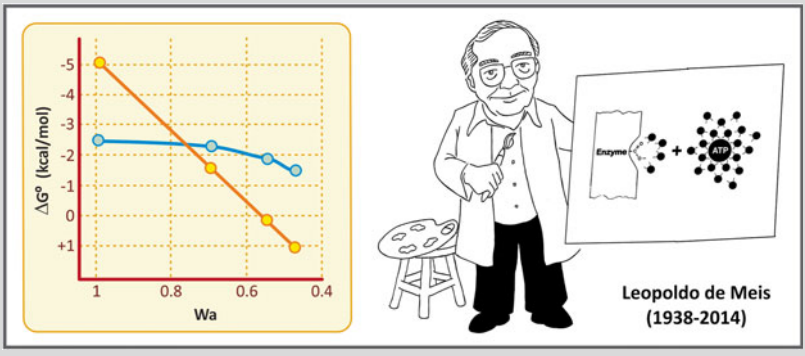
hexose that enters the pathway in its initials steps, four ATP molecules are synthesized at the end, yielding a positive balance of two ATP molecules for each hexose molecule that is metabolized. Moreover, consumption of ATP is mandatory to "force" the first steps to occur in terms of thermodynamics (see Box 4.2).

The triose phosphates with high potential of transferring their phosphoryl group are 1,3-bisphosphoglycerate and phosphoenolpyruvate (Fig. 6.4b). In the case of 1,3-bisphosphoglycerate, an anhydride bond links the carboxyl group to the phosphate, forming what is called an acyl phosphate. In the case of phosphoenolpyruvate, the double bond between carbons 2 and 3 favors the phosphoryl group transfer.

Additionally, the pathway includes an oxidative step in which the oxidation of a three-carbon intermediate is coupled to the reduction of the coenzyme nicotinamide adenine dinucleotide (NAD^+), generating NADH. Since the typical amount of NAD^+ in the cytoplasm is much lower than the amount of glucose metabolized, fermentation should end with a step leading to the reoxidation of the NADH molecule. The reaction that does this is the one that synthesizes the end product of the pathway, which in human cells is lactate (Fig. 6.4a).

Box 6.2: The Concepts of Energy-Rich Phosphate Compounds

In his classical article from 1941, Fritz Lipmann classified the phosphate compounds as "energy rich" and "energy poor." This classification was based on the conception that the energy released by the hydrolysis of these compounds was essentially determined by the nature of the chemical bond that links the phosphate to the molecule. Phosphoanhydride linkages generate "energy-rich" compounds, while phosphoester linkages generate "energy-poor" compounds. According to this concept, the free energy of hydrolysis of a phosphate compound would be determined essentially by the contribution of enthalpy. Due to the experimental approaches used at that time, the contribution of the environment in which the reaction takes place could not be taken into consideration, but new data obtained after 1970 introduced the idea that the energy of the hydrolysis of phosphate compounds would be also determined by the differences in their solvation energy and thus would vary greatly depending on the medium. This was found valid for compounds containing phosphoanhydride bonds, such as ATP, pyrophosphate, or acyl phosphates, but not for compounds in which the phosphate group is linked to the molecule through a phosphoester bond (see figure comparing the energy of hydrolysis of the phosphoanhydride bond of pyrophosphate with the phosphoester bond of glucose-6-phosphate as a function of water activity). Based on these observations, a new conception was formulated by the Brazilian scientist Leopoldo de Meis, according to which molecules containing phosphoanhydride bonds are susceptible to changes in the entropic energy (dependent on the solvation energy and thus on the environment), which contributes to the free energy involved in the reaction.



Effect of water activity (Wa) on the energy of hydrolysis of pyrophosphate (*yellow circles*) and glucose-6-phosphate (*blue circle*). Graph reproduced from de Meis in Calcium and Cellular Metabolism: Transport and Regulation, chapter 8. Plenum Press, NY, 1997

With this in mind, it is possible to understand the mechanism of ATP synthesis by ATP synthase presented in Sect. 6.2.4, in which the energy required in the reaction is not needed for the condensation of ADP and Pi, but for the ATP release from the catalytic site of the enzyme.

6.1.3 Glucose Fermentation Reactions

The first ten reactions of the fermentation process, starting with glucose and ending with the formation of two pyruvate molecules, are the same in many organisms that synthesize ATP anaerobically. The last step, which involves NADH reoxidation, is different depending on the organism, resulting in either lactate or ethanol and CO_2 as the end products. Furthermore, the reactions from glucose to pyruvate are also the same that occur when carbohydrates are used aerobically (see Sect. 7.4).

The pathway from glucose to pyruvate is named glycolysis and may be divided in two parts.

In the first part, two phosphorylation steps using ATP generate a hexose with two phosphoryl groups linked, the fructose-1,6-bisphosphate, which was the compound discovered by Harden and Young (see Sect. 6.1.1), and the first intermediate of fermentation to be identified, known as the Harden–Young ester. In the first phosphorylation step, the enzyme hexokinase catalyzes the conversion of glucose in glucose-6-phosphate, which is then isomerized to fructose-6-phosphate by the phosphohexose isomerase. The second phosphorylation step is catalyzed by the phosphofructokinase and occurs at the hydroxyl group on carbon 1 of fructose-6-phosphate, generating fructose-1,6-bisphosphate. Fructose-1,6-bisphosphate is then cleaved by the aldolase into two triose-phosphate molecules, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. These triose phosphates may interconvert in a reaction catalyzed by the triose-phosphate isomerase, with dihydroxyacetone phosphate forming glyceraldehyde-3-phosphate, the next intermediate of the pathway. Thus, in the end of this first phase, one glucose molecule is converted into two glyceraldehyde-3-phosphate molecules (Fig. 6.5). In the light of the points discussed in the previous section, it is important to note that all the phosphorylated compounds of this first part of glycolysis are phosphoesters.

In the second part of the pathway, each glyceraldehyde-3-phosphate molecule is oxidized in a NAD^+-dependent reaction followed by a phosphorylation step, in a reaction catalyzed by the glyceraldehyde-3-phosphate dehydrogenase, which forms 1,3-bisphosphoglycerate. Note that this phosphorylation step uses inorganic phosphate directly and not ATP as the phosphate donor. 1,3-bisphosphoglycerate is the first high-energy phosphorylated compound generated in the pathway. The oxidation of the aldehyde group of glyceraldehyde coupled to the phosphorylation reaction forms, instead of a free carboxyl group, an acyl phosphate, from which the phosphoryl group is transferred to ADP, generating ATP and 3-phosphoglycerate, in a reaction catalyzed by the phosphoglycerate kinase. Phosphoglycerate mutase converts 3-phosphoglycerate into 2-phosphoglycerate, which is then dehydrated by the enolase, forming the second compound in the pathway with high potential of transferring the phosphoryl group, the phosphoenolpyruvate (PEP). Then, another substrate-level phosphorylation step occurs, with the phosphoryl group of PEP transferred to ADP, generating ATP and pyruvate, a reaction catalyzed by the pyruvate kinase (Fig. 6.5).

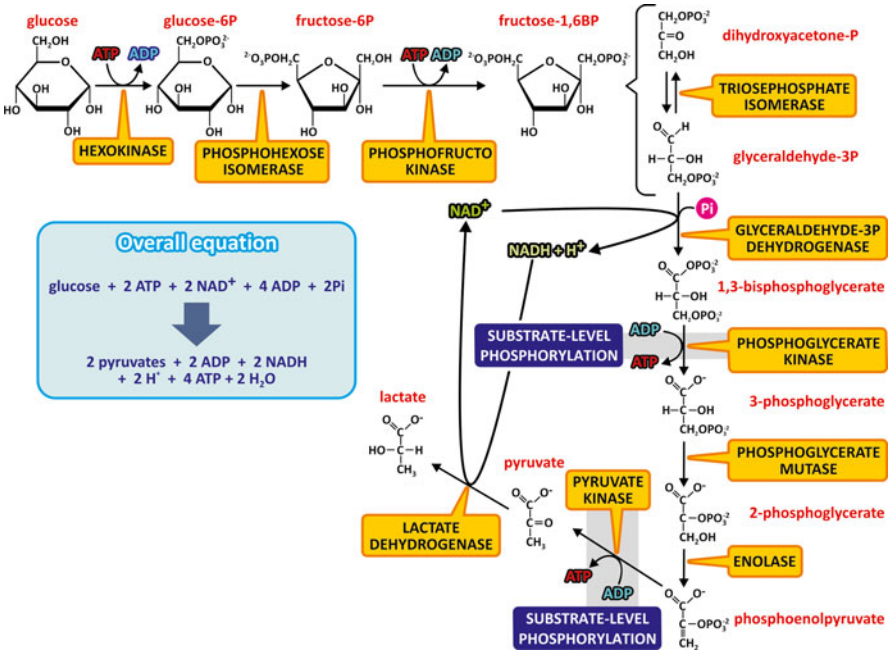


Fig. 6.5 Reactions of the fermentation of glucose to lactate. The first ten reactions consist in the metabolic pathway named glycolysis, which is also the pathway for the aerobic metabolization of carbohydrates. The last step occurs when pyruvate is not oxidized through the aerobic metabolism and NADH must be reoxidized. It should be noticed that each fructose-1,6-bisphosphate molecule leads to two glyceraldehyde-3-phosphate molecules, as dihydroxyacetone phosphate will be converted to glyceraldehyde-3-phosphate. The names of the enzymes are highlighted in *yellow boxes*. The steps that involve substrate-level phosphorylation are also indicated

The last step in the fermentation of glucose in human cells is the reduction of pyruvate to lactate, catalyzed by the lactate dehydrogenase, allowing the reoxidation of NADH (Fig. 6.5).

When glycolysis is the means of carbohydrate utilization with concomitant use of oxygen, instead of pyruvate being reduced in the last step of fermentation, it is completely oxidized to CO_2 in mitochondria (see Sect. 7.4).

6.2 Oxidative Phosphorylation: The Main Mechanism of ATP Synthesis in Most Human Cells

Oxidative phosphorylation accounts for 95 % of ATP synthesis in the human organism. This metabolic pathway is compartmentalized in mitochondria, the organelles that comprise most of the bioenergetic functions within the eukaryotic cell (see Box 6.3).

The mitochondria are unique organelles composed of two lipid membranes (Fig. 6.6). The outer mitochondrial membrane contains several porins, proteins that

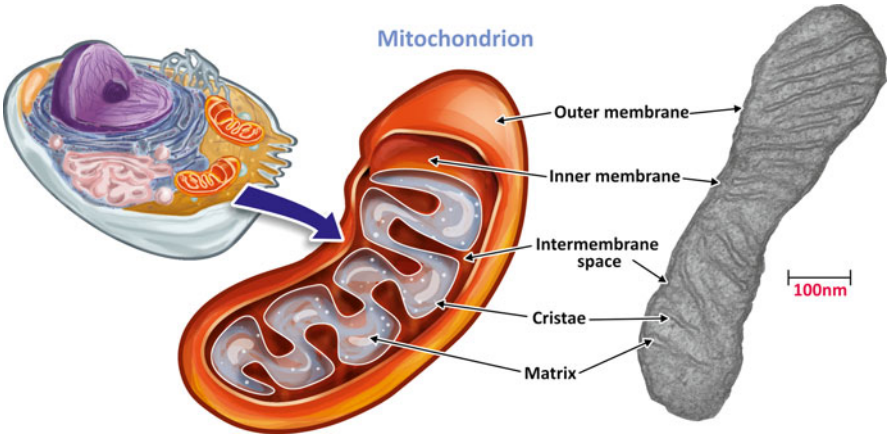


Fig. 6.6 The structure of a mitochondrion. Mitochondria are organelles formed by a permeable outer membrane and a very impermeable inner membrane with several convolution named cristae. The intramitochondrial medium forms the mitochondrial matrix. Mitochondrion transmission electron micrograph: courtesy from Prof. Marlene Benchimol

make membrane permeable to ions and small molecules (with molecular mass lower than 5 kDa). The inner membrane has a very high protein–lipid ratio and is rich in an unusual phospholipid, cardiolipin. It is very impermeable, with the transport of molecules and ions, including H^+, occurring only through specific proteins. This membrane has a very large surface area provided by several convolutions called mitochondrial cristae (Fig. 6.6). The inner membrane encloses the mitochondrial matrix, which comprises most of the enzymes of the oxidative metabolism, the mitochondrial ribosomes, tRNA, and several copies of the mitochondrial DNA (Fig. 6.6).

Box 6.3: The Endosymbiotic Theory for the Origin of Mitochondria

The incorporation of the mitochondria was an important event in the evolution of the eukaryotic cells. It is believed that it occurred more than 1.5 billion years ago through the invasion of a heterotrophic anaerobic cell by an aerobic bacterium. This is known as the endosymbiotic theory, which was postulated in the beginning of the twentieth century but was revived and better argued by Lynn Margulis, in the 1960s. Several genetic evidences suggest that the ancestral symbiont was an aerobic α-proteobacterium that consumed oxygen through a respiratory chain. The role of the anaerobic host in the symbiosis would be to make pyruvate accessible to the endosymbiont metabolism.

(continued)

Box 6.3 (continued)

On the other hand, evidence indicate that the role of the ancestral symbiont in the initial phase of the evolution of the mitochondria was to protect the anaerobic cell components from the toxic effects of oxygen through the activity of the last respiratory chain component, the enzyme cytochrome oxidase, which converts oxygen to water (see next sections of this chapter). In fact, the sharp increase in the oxygen tension around two billion years ago introduced a great threat for the anaerobic ancient cells, which did not have the detoxifying enzymes peroxidases, catalases, or superoxide dismutases that protect modern cells against the toxic effects of reactive oxygen species. Thus, the aerobic symbiont would function as an oxygen scavenger inside the host cell. With time, evolution of host genomes probably contributed with new functions to the symbiont, including the ATP/ADP transporter, transforming it into an organelle with an ATP-exporting function.

6.2.1 A Historical Perspective of the Understanding of Cellular Respiration

Since Lavoisier's experiments in the eighteenth century, which correlated respiration to the combustion of organic matter, both processes involving oxygen consumption and CO_2 release together with heat production, the concept that aerobic metabolism proceeds with the direct reaction between oxygen and the organic compounds in the body was established. However, the connection between metabolic oxidative reactions and oxygen consumption in respiration remained illusive for a long time.

In the first decades of the twentieth century, there was a serious controversy regarding the mechanisms of biological oxidations in aerobic metabolism (Fig. 6.7).

On one side, Heinrich Wieland postulated, together with Torsten Thumberg, that in the biological oxidations, the reactions catalyzed by the dehydrogenases "activated" some hydrogen atoms of the metabolic intermediates, making them labile to be transferred to a hydrogen acceptor. It is important to mention that their hypothesis, although incomplete by not taking into account the role of oxygen, correctly supported the concept that free oxygen does not directly combine with carbon to form CO_2.

On the opposite side was Otto Warburg (Box 6.4), who defended the oxygen-activating hypothesis, in which he argued that the dehydrogenase concept was unnecessary. In this hypothesis, Warburg postulated that the oxidation of all metabolites was catalyzed by an iron-containing enzyme, which he named *Atmungsferment* (meaning oxygen-transferring enzyme or respiratory enzyme) and in which the iron atom was oxidized to its ferric state (Fe^{3+}) by oxygen and reduced back to its ferrous form (Fe^{2+}) after reaction with organic substances.

The key to solve the Wieland–Warburg controversy was provided by David Keilin, who working as entomologist and parasitologist changed biochemistry with his findings (Fig. 6.7).

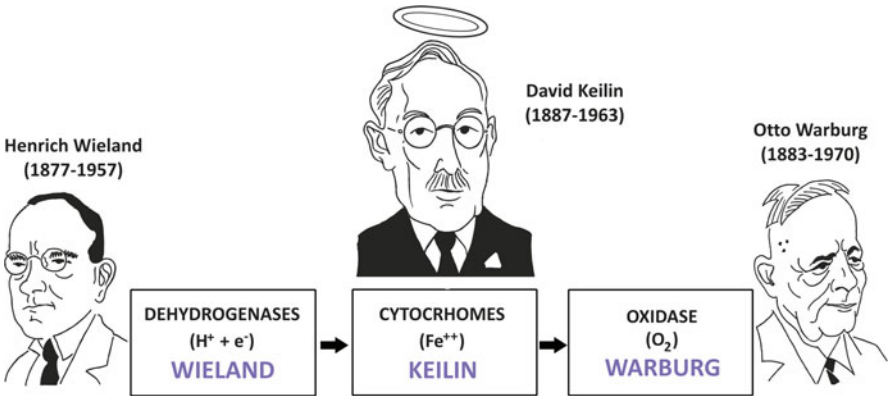
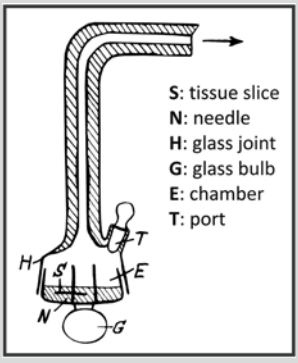


Fig. 6.7 Solution of Wieland–Warburg controversy with the discovery of the cytochromes by Keilin. Wieland postulated that the reactions catalyzed by the dehydrogenases "activated" some hydrogen atoms of the metabolic intermediates, making them labile to be transferred to a hydrogen acceptor while Warburg defended the oxygen-activating hypothesis, in which biological oxidations were catalyzed by an iron-containing enzyme. Keilin proposed that the cytochromes connected the dehydrogenases and the oxidase, being alternately reduced by the dehydrogenases and oxidized by oxygen through the Warburg enzyme

Box 6.4: The Diversity of Otto Warburg's Contributions for Science

Otto Warburg was a very active and interdisciplinary scientist, giving outstanding contributions to different fields in science, including respiration, photosynthesis, and cancer cell metabolism.

Warburg strongly defended the use of quantitative methods and worked on the improvement of the instruments to get reliable measurements. Using manometric techniques, he developed an apparatus, the Warburg respirometer (see figure below), to quantify O_2 uptake by thin slices of a tissue, through the changes in the chamber pressure, measured by the connection of a manometer



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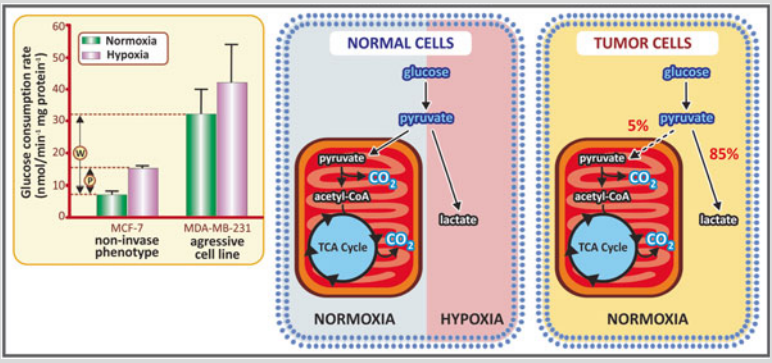
Box 6.4 (continued)

to the end of the glass joint (H). The apparatus also allowed the measurements of CO_2 emission by adding, for example, potassium hydroxide in the chamber (E) to precipitate CO_2. Due to his pioneering studies on cellular respiration in the beginning of the twentieth century, in which he proposed the existence of an iron-containing respiratory enzyme (the Warburg *Atmungsferment*), he was awarded the Nobel Prize in Physiology or Medicine in 1931.

Warburg also dedicated much time of his life investigating cancer cell metabolism. Studying different types of cancer cells in the decade of 1920, Warburg made a very intriguing observation: he found a behavior that was the opposite of the Pasteur effect (the inhibition of fermentation by O_2; see Sect. 6.1.1). Warburg showed that cancer cells produced lactic acid from glucose even under aerobic conditions, which is known as the Warburg effect (see figure below). This was firstly interpreted as a consequence of a damaged respiration in cancer cells, but now we know that the Warburg effect occurs due to alterations in the regulation of glycolysis in tumorigenesis.



Pasteur (P) and Warburg (W) effects. Graph in the left reproduced by permission from Macmillan Publishers Ltd: Gatenby & Gillies. Nat. Rev. Cancer 4:891–899, 2004

Studying the physiology of insects, Keilin rediscovered a substance that has been described more than a century before by Charles A. MacMunn, but was forgotten after it has been considered a hemoglobin contaminant present in MacMunn's preparations (see Box 6.5). Keilin showed that it was not hemoglobin and named this substance "cytochrome" (meaning cellular pigment) because he had found it in cells of many different organisms, such as insects, worms, yeast, and plants, and due to

its characteristic absorption spectrum containing four distinct bands, which he named *a*, *b*, *c*, and *d*. Keilin proposed that this pigment acted as the link between the Wieland dehydrogenases and the Warburg oxidase, being alternately reduced by the dehydrogenases and oxidized by oxygen through the Warburg enzyme.

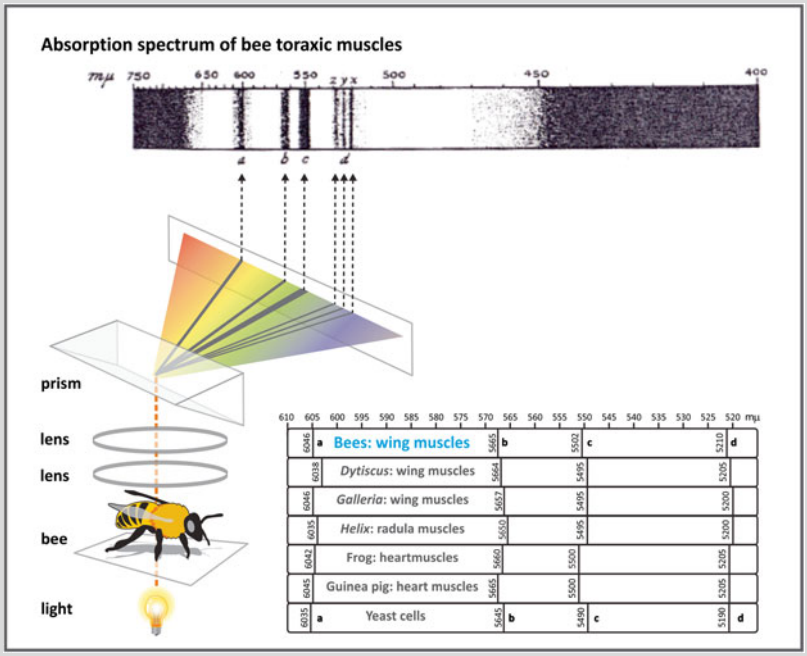
Keilin's experiments provided a great advance in the comprehension of the energy transformations in aerobic organisms, establishing the concept of what we now know as the respiratory chain, a series of membrane-associated redox carriers that transfer the electrons from the metabolic substrates to molecular oxygen. However, the process by which this sequence of redox reactions is coupled to energy storing into the cells was still unknown at that time.

Box 6.5: The Discovery of the Cytochromes by David Keilin

The basic features of the respiratory chain were established between the 1920s and 1930s by the entomologist and parasitologist David Keilin. In 1925, he published his first paper in this field, entitled "*On cytochrome, a respiratory pigment, common to animals, yeast, and higher plants*" (Keilin. Proc. R. Soc. Lond. B 98:312–339, 1925), marking a new phase in the studies of the biological oxidations. It is interesting to revisit the reflexions of E. F. Hartree, who worked with Keilin on this subject, published in the periodic Biochemical Education, in 1973, in which he comments the history of the discovery of the cytochromes: "In these days, when the world of science is under pressure to organize for the pursuit of practical ends, when the scale of scientific endeavour is making the lone furrow an anachronism, it is salutary to recall the simple-handed achievements of men of science stimulated solely by an urge to understand the living world. The discovery of cytochromes is a notable example of such achievements, not only because the disarming simplicity of the experimental approach by the discoverer, David Keilin, but also because the discovery came at a critical moment. It resolved a serious dilemma that was impeding the evolution of biochemistry from an untidy and rather primitive branch of chemistry into a major scientific discipline: more succinctly the transformation of Bio-Chemistry into Biochemistry." At that time, Keilin was interested in studying the adaptations of a fly larva that parasitizes the stomach of horses. Using a microspectroscope to compare the absorption spectra of the larvae and the adult thoracic muscle, he found the presence of four sharp absorption bands in the spectrum of the adult muscle, which were very different from the bands seen for hemoglobin and oxyhemoglobin (see in the figure the results published in the 1925's paper with a schematic representation of the experiment). Intrigued by this absorption pattern, he examined many other organisms, including different insects, yeast and other microorganisms, plants, and animal tissues, finding always the same absorption spectra with the four bands.

(continued)

Box 6.5 (continued)



A more intriguing observation was that when a yeast suspension was submitted to vigorous shaking, the bands disappeared, appearing again after a period of rest. Keilin's interpretation of this phenomenon was that the pigment, which he named cytochrome, became oxidized during the shaking in the air, leading to the disappearance of the bands. After interruption of the shaking, yeast respiratory activity led to reduction of the pigment, which in the reduced form displayed the four-banded absorption spectrum. Using insects, Keilin observed strikingly similar results: the absorption band pattern observed in the thoracic muscle was strong when the wings were stimulated to vibrate but disappeared after the movement stopped. Thus, Keilin concluded that the cytochrome was acting as a respiratory carrier linking oxygen and the oxidizable metabolic intermediates.

One of the landmarks in the discovery of this missing link was the article published by Belitser and Tsybakova in 1939, showing a direct correlation between oxygen consumption in cellular respiration and phosphate esterification, as it can be seen in Fig. 6.8, a reproduction of the original figure of this article.

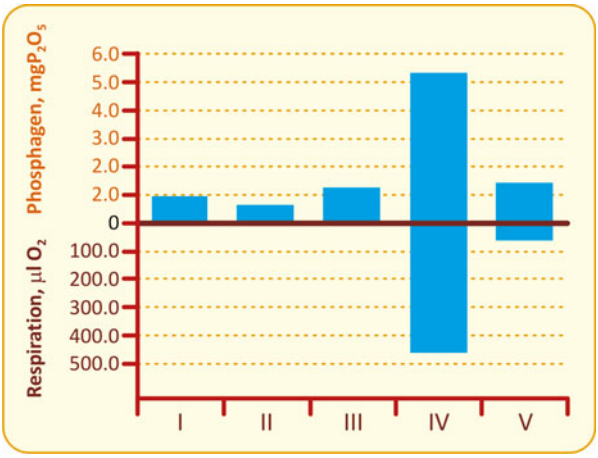


Fig. 6.8 Correlation between phosphate incorporation (*top*) and O_2 consumption (*bottom*) in a pigeon muscle preparation in the following conditions: (I) before any incubation; (II) in the absence of O_2 (in a N_2 atmosphere) without addition of a respiratory substrate; (III) in the presence of pyruvate but in the absence of O_2; (IV) in the presence of pyruvate and O_2; and (V) in the presence of O_2 but in the absence pyruvate. Figure adapted from Belitser & Tsybakova. Biokhimia 4:516–535, 1939

After this, years and years were spent searching a high-energy intermediate, which, in analogy to the substrate-level phosphorylation process already known to be the mechanism of ATP synthesis in fermentation (see Sect. 6.1.2), would couple the redox process to ATP synthesis. This intermediate had never been found, and how oxidative phosphorylation occurred remained one of the most challenging questions of Biochemistry for a long time. The basic principles behind this process could only be understood after the proposal of the chemiosmotic hypothesis by Peter Mitchell (see next section).

6.2.2 An Overview of Oxidative Phosphorylation Process

In the oxidative reactions of catabolism, the electrons removed from the metabolic intermediates are transferred to two major electron carrier coenzymes, nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD), which are converted to their reduced forms, NADH and $FADH_2$ (see Chap. 7). These reactions are catalyzed by dehydrogenases, as firstly postulated by Wieland and Thumberg (see Sect. 6.2.1).

Oxidative phosphorylation depends on the electron transport from NADH or $FADH_2$ to O_2, which is reduced to H_2O. Electron transport occurs through a number of protein complexes associated to the inner mitochondrial membrane. Some of these protein complexes contain cytochromes (the Keilin's pigment) as part of their structures. These cytochromes are in fact proteins that contain a heme prosthetic

group. Heme is a complex ring structure named protoporphyrin that binds an iron atom. As detected by Keilin, there are different classes of cytochromes that contain different types of heme (Fig. 6.9), distinguishable by their characteristic absorption spectra. Heme oxidation leads to a decrease in light absorption, explaining the observations made by Keilin in his experiments (see Box 6.5). The heme groups in the cytochromes *a* and *b* are tightly but not covalently bound to the protein, while in cytochrome *c* the heme is covalently linked to specific Cys residues of the protein.

The structures of respiratory protein complexes are now known with detail, as it will be explained in the next section, but one can safely say that the concept and the principles of their role in electron transport were established by Keilin after the discovery and the study of the cytochromes, which he defined as "oxidation–reduction catalysts" (see Sect. 6.2.1).

The mechanism by which the electron transport is coupled to ATP synthesis was proposed by Peter Mitchell, in 1961, in his revolutionary chemiosmotic theory. Initially it was difficult to be accepted, since most of the scientists in the field believed that a high-energy intermediate would link oxidation to phosphorylation reactions (as occurs in the substrate-level phosphorylation process; see Sect. 6.1.2). However, the chemiosmotic hypothesis was proved to be correct, and Mitchell was awarded the Nobel Prize in Chemistry in 1978.

The chemiosmotic theory postulates that the electron transfer through the respiratory protein complexes is coupled to proton (H^+) pumping across the proton-impermeable mitochondrial inner membrane, from the mitochondrial matrix to intermembrane space. H^+ pumping generates what Mitchell called the proton-motive force, the simultaneous effect of the pH gradient across the membrane and transmembrane electrical potential that drives the ATP synthesis from ADP and Pi (Fig. 6.10).

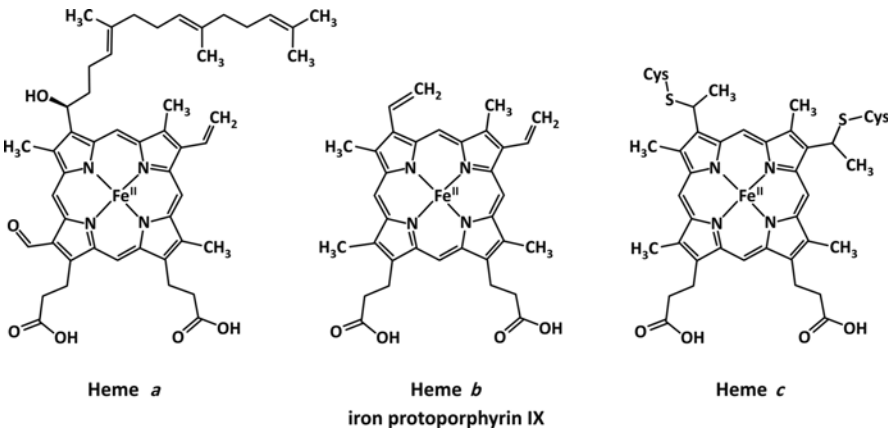


Fig. 6.9 The distinct types of heme prosthetic groups of the cytochromes: heme *a*, which has a long isoprenoid tail attached to the porphyrin ring; heme *b*, which is a protoporphyrin IX; and heme *c*, which is covalently bound to Cys residues in the polyprotein chain of the cytochrome *c*

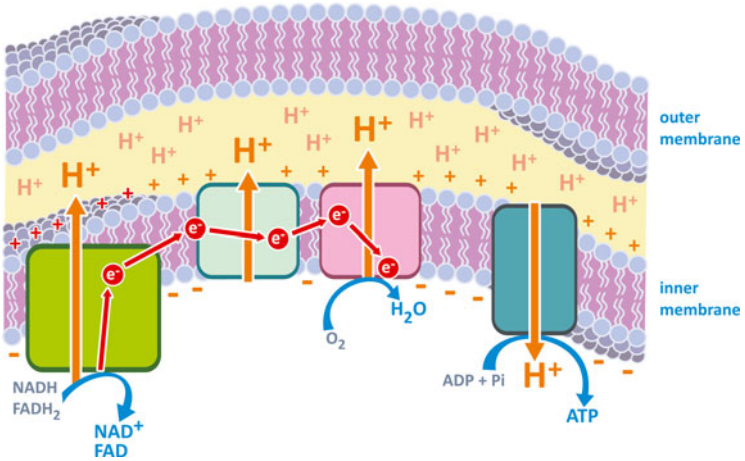


Fig. 6.10 Schematic representation of the general mechanism of ATP synthesis in oxidative phosphorylation. Electrons are transferred from NADH or $FADH_2$ through a number of protein complexes associated to the inner mitochondrial membrane to the final acceptor oxygen, which is reduced to H_2O. Electron transport is coupled to H^+ pumping across mitochondrial inner membrane, generating a pH gradient and a transmembrane electrical potential, which is the driving force for ATP synthesis by ATP synthase. Note that this is a simplified scheme in which the NAD- or FAD-associated complexes are represented as one entity, although they are different protein complexes. However, it is important to point out that electron transport through FAD-associated complex is not coupled to H^+ pumping across mitochondrial membrane, as detailed in the next section

The H^+ pumping occurs through specific protein segments that are part of some of the respiratory complexes. The ATP synthesis is catalyzed by another protein complex in the mitochondrial membrane, the ATP synthase, through which the H^+ ions return to the matrix (Fig. 6.10).

6.2.3 The Electron Transport System

The electron transport complexes are integral membrane proteins that contain attached chemical groups (flavins, iron–sulfur groups, heme or cooper ions) capable of accepting and donating electrons (see detailed structures in the next section).

6.2.3.1 The Sequence of Electron Transfer Between the Electron Carrier Groups

A form to deduce the sequence in which the electrons are transferred between the carriers is to compare the reduction potential of each individual electron carrier group (Table 6.1). The reduction potential is a measure of how "easy" is for a molecule to accept an electron.

Table 6.1 Reduction potential of respiratory electron carriers

Reaction	Reduction potential (V)
$2H^+ + 2e^- \rightarrow H_2$	–0.414
$NAD + H^+ + 2e^- \rightarrow NADH$	–0.320
NADH dehydrogenase (FNM) $+ 2H^+ + 2e^- \rightarrow$ NADH dehydrogenase ($FNMH_2$)	–0.300
Ubiquinone $+ 2H^+ + 2e^-$ → ubiquinol	0.045
Cytochrome *b* (Fe^{3+}) $+ e^-$ → cytochrome *b* (Fe^{2+})	0.077
Cytochrome c_1 (Fe^{3+}) $+ e^-$ → cytochrome c_1 (Fe^{2+})	0.220
Cytochrome *c* (Fe^{3+}) $+ e^-$ → cytochrome *c* (Fe^{2+})	0.254
Cytochrome *a* (Fe^{3+}) $+ e^-$ → cytochrome *a* (Fe^{2+})	0.290
Cytochrome a_3 (Fe^{3+}) $+ e^-$ → cytochrome a_3 (Fe^{2+})	0.350
½ $O_2 + 2H^+ + 2e^- \rightarrow H_2O$	0.817

The sequence deduced by the reduction potential was confirmed experimentally. Keilin in his first experiments on the cytochromes (see Box 6.5) observed that the bands do not appear nor disappear simultaneously. From this observation, he deduced that what he firstly named simply cytochrome was a mixture of pigments containing three components, designated cytochromes *a*, *b*, and *c*. Afterward, it was possible to distinguish two components in what was considered as cytochrome *a*, whose absorption bands were superimposed. One of them, the cytochrome *a*3, is the last component in the chain, being directly oxidized by oxygen. Furthermore, the use of specific inhibitors of the electron transport allowed the complete sequence to be determined, confirming that the sequence follows the order of increasing reduction potential, as expected.

6.2.3.2 The Organization of the Respiratory Complexes in the Inner Mitochondrial Membrane

Although Keilin's experiments allowed the determination of the sequence through which the electrons flow in the respiratory chain, nothing was known at that time about how the electron-transferring groups were organized. Keilin always worked with a grinded heart muscle preparation that later was identified as submitochondrial particles, which are actually vesicles of the inner mitochondrial membrane. At the end of the 1940s, the group of David Green was able to fractionate four components of the respiratory chain, which they named Complexes I, II, III, and IV. These components corresponded to four integral membrane protein complexes, the NADH/ubiquinone oxidoreductase (or NADH dehydrogenase), the succinate/ubiquinone oxidoreductase (or succinate dehydrogenase), the ubiquinone/cytochrome *c* oxidoreductase (or cytochrome *bc*1 complex), and the cytochrome *c* oxidase, respectively (Fig. 6.11).

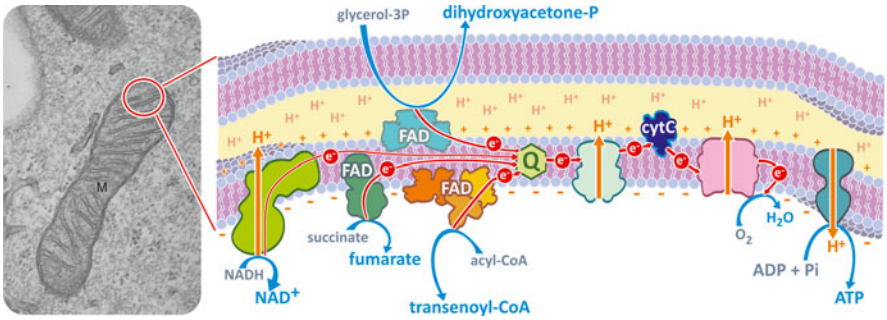


Fig. 6.11 Schematic representation of the electron transfer complexes in the inner mitochondrial membrane. The electrons flow from NADH to O_2 through three protein complexes: NADH/ubiquinone oxidoreductase (*light green*), ubiquinone/cytochrome *c* oxidoreductase (*light blue*), and cytochrome *c* oxidase (*pink*). The electron transference from NADH/ubiquinone oxidoreductase to ubiquinone/cytochrome *c* oxidoreductase is mediated by the membrane-soluble molecule ubiquinone (*green hexagon* labeled with a Q), and the transference from ubiquinone/cytochrome *c* oxidoreductase to cytochrome *c* oxidase is mediated by the small protein cytochrome *c* (*dark blue* labeled with cytC). O_2 is also reduced by electrons coming from $FADH_2$, which is linked to FAD-dependent dehydrogenases, such as succinate/ubiquinone oxidoreductase (*dark green*), the acyl-CoA dehydrogenase (*orange*), and the glycerol-phosphate dehydrogenase (*blue*). The electrons from $FADH_2$ are transferred to ubiquinone and then flow to O_2 through ubiquinone/cytochrome *c* oxidoreductase and cytochrome *c* oxidase, as described for the electrons transferred from NADH. The electron transferring through NADH/ubiquinone oxidoreductase, ubiquinone/cytochrome *c* oxidoreductase, and cytochrome *c* oxidase is coupled to H^+ pumping to the intermembrane space. The H^+ returns to the matrix through the enzyme ATP synthase, driving ATP synthesis. The transmission electron micrograph in the *left* shows a cell thin section with a mitochondrion (courtesy from Prof. Marlene Benchimol)

Now we know that in addition to these four protein complexes, there are two other proteins that participate in the transfer of electrons from metabolic substrates to the respiratory chain, the acyl-CoA dehydrogenase and the glycerol-phosphate dehydrogenase (Fig. 6.11).

Electron transport between the respiratory complexes occurs through two more mobile electron carriers, the ubiquinone (also called coenzyme Q), a very hydrophobic molecule with high mobility in the mitochondrial membrane (independently on its protonation state), and cytochrome *c*, a small protein associated to the outer face of the inner mitochondrial membrane (Fig. 6.11).

NADH formed in metabolic NAD^+-dependent oxidative reactions is a water-soluble electron carrier that reversibly associates to specific dehydrogenases. The electrons are transferred from NADH to O_2 through three protein complexes: NADH/ubiquinone oxidoreductase, ubiquinone/cytochrome *c* oxidoreductase, and cytochrome *c* oxidase.

The electron carrier FAD is usually covalently attached to a FAD-dependent dehydrogenase. This type of enzyme includes the succinate dehydrogenase, the acyl-CoA dehydrogenase, and the glycerol-phosphate dehydrogenase. In the reac-

tions catalyzed by these three FAD-dependent enzymes, FAD is reduced to $FADH_2$, whose electrons are then transferred to O_2 through ubiquinone/cytochrome *c* oxidoreductase and cytochrome *c* oxidase, as described for NADH/ubiquinone oxidoreductase electrons (Fig. 6.11).

The numbering of the four firstly identified electron transport complexes from I to IV and the concept that they form a chain, as inferred by the usually used name of "electron transport chain" or "respiratory chain," give rise to a not entirely correct idea that the electron transport complexes are arranged sequentially and that the electron transport occurs through a linear pathway.

A more appropriated terminology emerges from the concept of a convergent "electron transport system," as proposed by Erich Gnaiger, in which electrons either from NADH via Complex I or from $FADH_2$ through three different FAD-associated complexes, the Complex II, the electron-transferring flavoprotein (ETF), or the glycerol-phosphate dehydrogenase (GpDH), converge to ubiquinone, in what he named the "Q-junction" (Fig. 6.12). After this point of convergence, the electrons flow to oxygen through a "linear" pathway composed of Complex III, cytochrome *c*, and Complex IV.

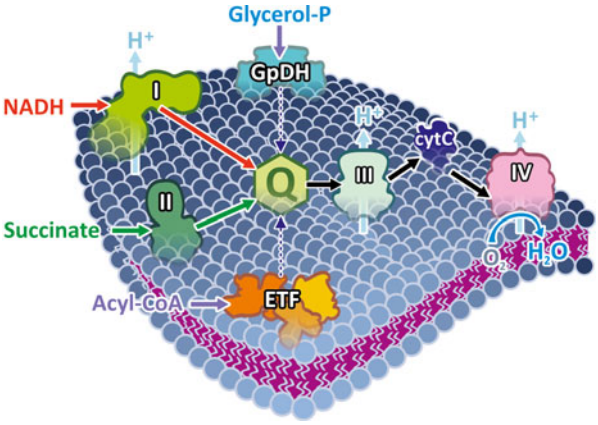


Fig. 6.12 Convergent electron transport system. Ubiquinone receives the electrons from four different protein complexes: NADH/ubiquinone oxidoreductase (or Complex I), succinate/ubiquinone oxidoreductase (or Complex II), ETF/Q oxidoreductase (ETF); and glyceraldehyde dehydrogenase (GpDH). NADH comes from NAD-dependent dehydrogenases, mainly glyceraldehyde-3-phosphate dehydrogenase, pyruvate dehydrogenase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and malate dehydrogenase. $FADH_2$ is linked to succinate/ubiquinone oxidoreductase, GpDH, or acyl-CoA dehydrogenase, which in turn is associated to ETF. Then, electrons are transported to O_2 through a "linear" pathway composed of ubiquinone/cytochrome *c* oxidoreductase (or Complex III) and cytochrome *c* and cytochrome *c* oxidase (or Complex IV)

6.2.3.3 The Structure of the Electron-Transferring Components

A detailed description of each electron-transferring component of the respiratory chain is presented below as these are paradigmatic cases of structure/function correlation.

NADH/ubiquinone oxidoreductase (or Complex I) is composed of 45 polypeptide chains associated to several electron-transferring groups: a flavin nucleotide (FMN) and many iron–sulfur (Fe–S) centers. The complete crystal structure of NADH/ubiquinone oxidoreductase is only available for the simpler prokaryotic enzyme (Fig. 6.13a), but the high degree of sequence conservation suggests that the bacterial

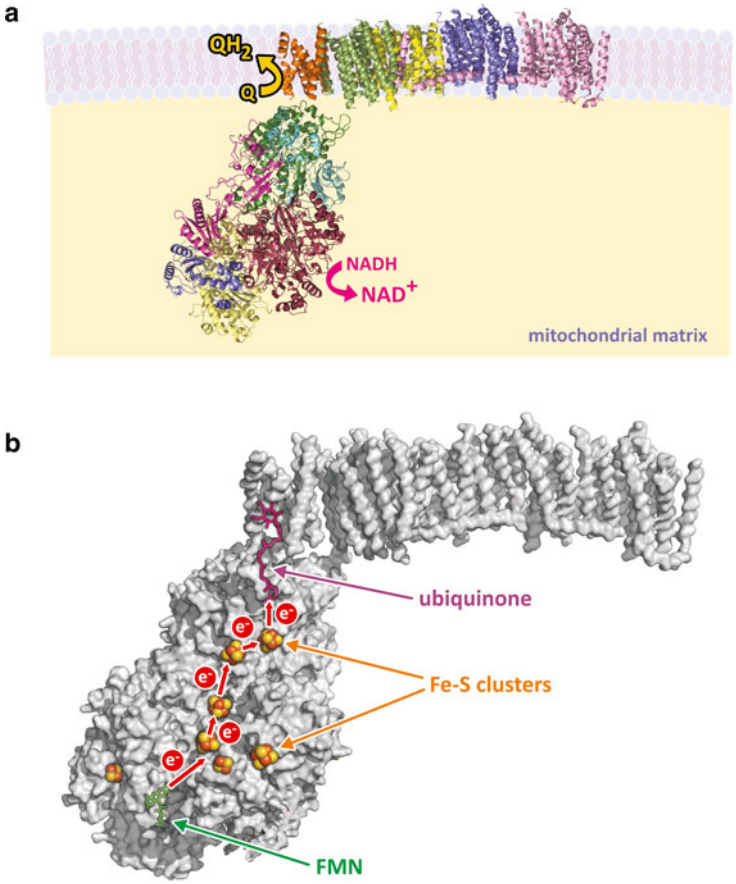


Fig. 6.13 (**a**) Structure NADH/ubiquinone oxidoreductase complex from *Thermus thermophilus* (PDB 3M9S), with each subunit colored differently. This complex catalyzes the oxidation of NADH to NAD^+, with the reduction of ubiquinone (Q) to ubiquinol (QH_2). (**b**) The transfer of electrons (e^-) from NADH to ubiquinone (shown in *magenta sticks*), flowing through FMN (shown in *green sticks*) and iron–sulfur clusters (*orange* and *yellow spheres*), is represented by the *red arrows* over the protein surface map. Electron transfer is coupled to the translocation of four protons

enzyme represents a minimal model of human enzyme. The electrons removed from NADH flow through FMN and then to the Fe–S groups to finally reduce ubiquinone to ubiquinol (Fig. 6.13b). The electron transfer through the enzyme components drives the transport of four H^+ from the matrix to the intermembrane space.

Succinate/ubiquinone oxidoreductase (or Complex II) is an FAD-dependent mitochondrial membrane enzyme that catalyzes the oxidation of succinate to fumarate, a reaction of the tricarboxylic acid (TCA) cycle, the pathway that accounts for the complete oxidation of acetyl-CoA, which, in turn, is the convergent product of the degradation pathways of sugars, lipids, and some amino acids (see Sect. 7.2). This enzyme is usually referred as succinate dehydrogenase, but since the oxidation of succinate to fumarate is coupled to electron transference to ubiquinone, succinate/ubiquinone oxidoreductase is a more precise denomination. The enzyme contains four polypeptide chains, a catalytic heterodimer composed of subunit A, containing a covalently bound FAD, and subunit B, containing three iron–sulfur clusters, and two transmembrane polypeptides that anchor the enzyme in the mitochondrial membrane and where a heme *b* group is bound (Fig. 6.14).

Acyl-CoA dehydrogenase (ACAD) catalyzes the first step of mitochondrial fatty acid oxidation: the conversion of an acyl-CoA to trans-2,3-enoil-CoA with the

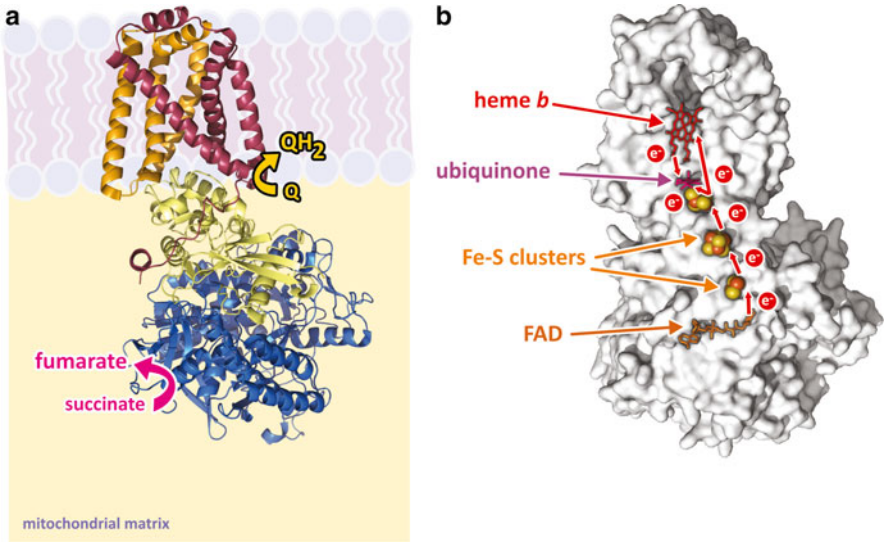


Fig. 6.14 (**a**) Structure of succinate/ubiquinone oxidoreductase complex from porcine heart (PDB 1ZOY). The FAD binding protein, or subunit A, is shown in *blue*; the iron–sulfur protein, or subunit B, is shown in *light yellow*; and the transmembrane proteins are shown in *pink* and *orange*. The enzyme catalyzes an FAD-dependent oxidation of succinate to fumarate, with the concomitant reduction of ubiquinone (Q) to ubiquinol (QH_2). (**b**) The electron (e^-) transfer pathway from FAD (shown in *orange sticks*) to heme *b* (shown in *red sticks*), flowing through the iron–sulfur groups (*orange* and *yellow spheres*) to finally reduce the ubiquinone (shown in *magenta sticks*), is represented by the *red arrows* over the protein surface map

reduction of the enzyme-bound FAD coenzyme (see Sect. 7.4.4). ACADs associate to the electron-transferring flavoprotein (ETF), which reoxidizes ACAD-bound $FADH_2$. ETF then transfers the electrons to the ETF/Q oxidoreductase that in turn reduces ubiquinone after electron transport through the Fe–S centers (Fig. 6.15). There are five isoforms of this enzyme showing distinct specificity for the fatty acyl chain length. The very-long-chain acyl-CoA dehydrogenase (VLCAD) forms homodimers of 67 kDa subunits bound to the inner mitochondrial membrane through the 180 last residues of the C-terminal. These residues are lacking in the long-, medium-, and short-chain acyl-CoA dehydrogenases (LCAD, MCAD, and SCAD, respectively), isoforms that are soluble homotetramers with 45 kDa subunits.

Glycerol-phosphate dehydrogenase (GpDH) is a dimeric enzyme associated to the outer face of the inner mitochondrial membrane that oxidizes glycerol-3-phosphate to dihydroxyacetone phosphate (DHAP) with reduction of the enzyme-bound FAD that mediates the transfer of the electrons to ubiquinone (Fig. 6.16).

Ubiquinone is a lipid-soluble benzoquinone with an isoprenoid tail, which in mammals is composed of 10 isoprenyl units, making the molecule very hydrophobic and thus soluble in membranes (Fig. 6.17). It is reduced to ubiquinol after accepting two electrons and two H^+ from NADH/ubiquinone oxidoreductase, succinate/ubiquinone oxidoreductase, ETF/Q oxidoreductase, or GpDH and diffuses in the membrane, reaching ubiquinone/cytochrome *c* oxidoreductase to which the electrons are transferred.

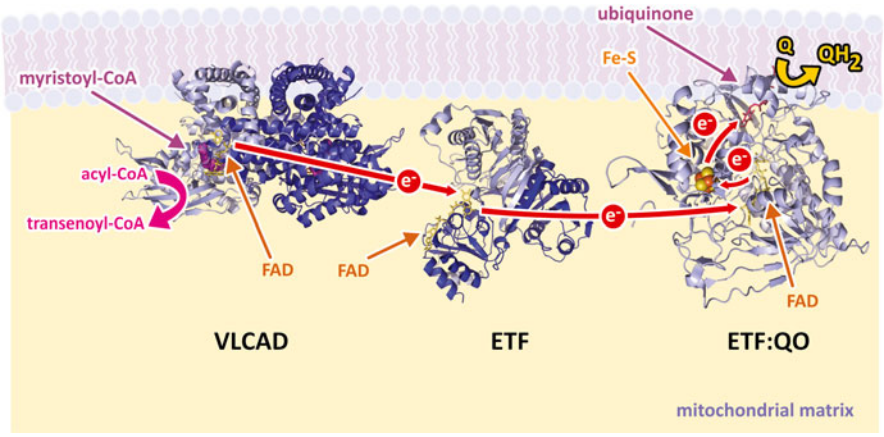


Fig. 6.15 Representation of the multistep electron transfer from an acyl-CoA (oxidized to transenoyl-CoA) to ubiquinone (Q, reduced to ubiquinol, QH_2). The known structures used as examples are the human VLCAD dimer (PDB 3B96) complexed with the substrate myristoyl-CoA (shown in *pink spheres*); the human ETF (PDB 1EFV); and the ETF/QO from pig liver (PDB 2GMH) complexed with ubiquinone (shown in *magenta sticks*). The electrons (e^-) are transferred through each enzyme-bound FAD (shown in *orange sticks*) to the iron–sulfur center (shown in *orange* and *yellow spheres*) in ETF/QO to finally reduce the ubiquinone

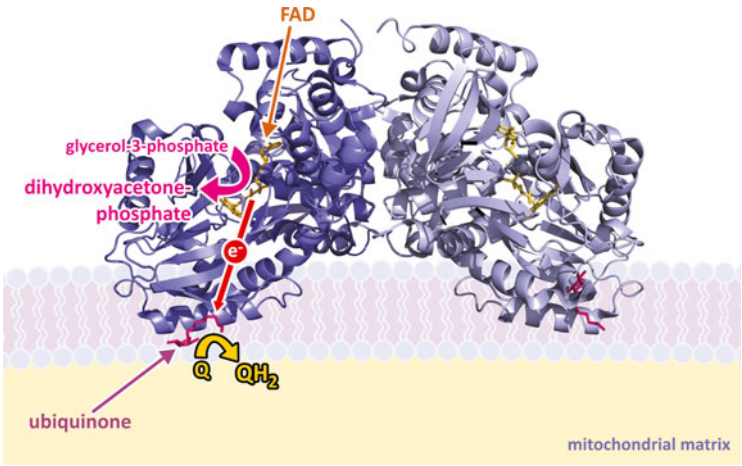


Fig. 6.16 Structure of the GpDH dimer from *E. coli* (PDB 2QCU), showing the enzyme-bound FAD (in *orange sticks*), which transfers electrons (e^-) to ubiquinone (Q, reduced to ubiquinol, QH_2; molecular structure shown in *magenta sticks*)

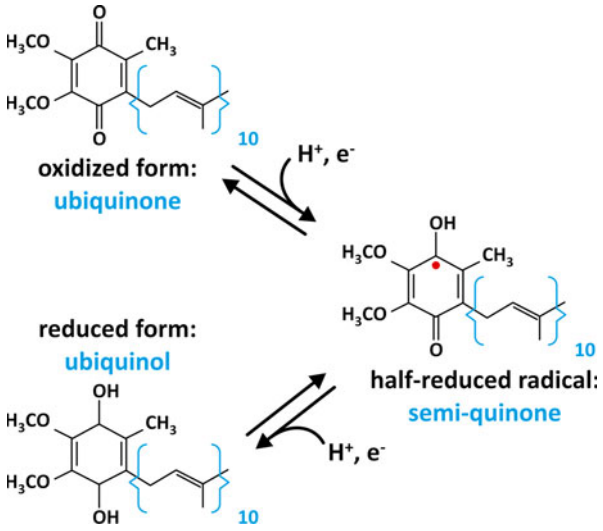


Fig. 6.17 Structures of the redox forms of coenzyme Q: ubiquinone is the oxidized form that is completely reduced to ubiquinol by accepting two electrons and two H^+ in the reactions catalyzed by NADH/ubiquinone oxidoreductase, succinate/ubiquinone oxidoreductase, ETF/Q oxidoreductase, or GpDH. Ubiquinol is reoxidized by the ubiquinone/cytochrome *c* oxidoreductase. The reduction of ubiquinone and the oxidation of ubiquinol involves an intermediate step in which a half-reduced semiquinone radical ($^{\bullet}Q^-$) is formed. The implications of this will be discussed in Sect. 6.2.5

Ubiquinone/cytochrome *c* oxidoreductase (or Complex III) transfers the electrons from ubiquinol to cytochrome *c* with the coupled transport of four H^+ from the matrix to the intermembrane space. This protein complex is a dimeric structure with each monomer being a complex assembly of 11 polypeptide chains. The electrons are transported through three functional groups associated to each monomer: cytochrome *b* containing two *b*-type heme groups, an Fe–S center, and cytochrome *c*1 containing one *c*-type heme group (Fig. 6.18).

Cytochrome *c* is a monomeric protein containing one heme group (Fig. 6.19). This protein is located in the intermembrane space in close association to the inner mitochondrial membrane. It is reduced by ubiquinone/cytochrome *c* oxidoreductase and reoxidized by cytochrome *c* oxidase.

Cytochrome *c* oxidase (or Complex IV) is a dimeric complex of 13 subunit monomers that transfers electrons from cytochrome *c* to O_2, forming H_2O. It contains two Cu ions associated to the SH groups of two Cys of the subunit that receives the electrons from cytochrome *c* and transfers them to an *a*-type heme group that in turn transfers the electrons to another heme *a* group, heme a_3, this one associated to another Cu ion, which finally transfers the electrons to O_2 (Fig. 6.20).

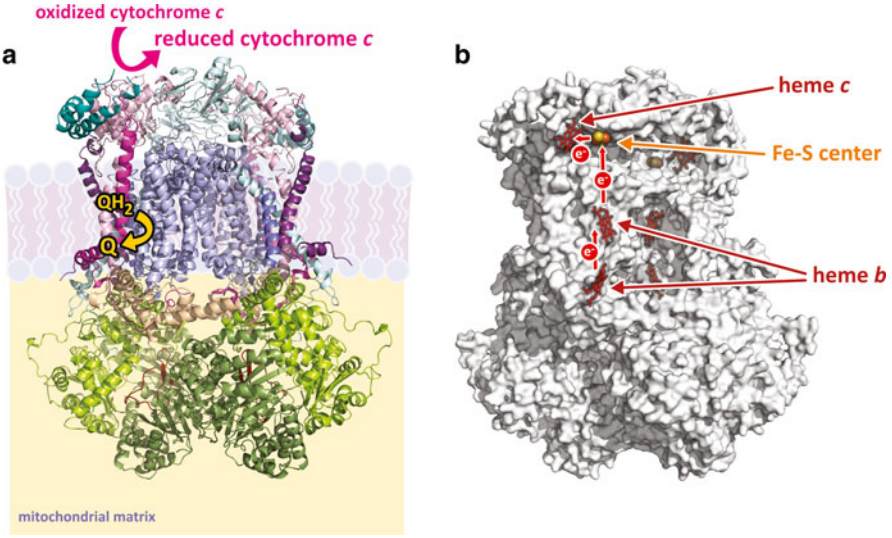


Fig. 6.18 (**a**) The complete dimeric structure of the bovine ubiquinone/cytochrome *c* oxidoreductase (PDB 1BE3) with the 11 subunits colored differently. (**b**) The electron (e^-) transfer pathway from ubiquinol to cytochrome *c*, flowing through the heme *b* groups (shown in *red sticks*) of the cytochrome *b* subunit (shown in *light purple* in A), the iron–sulfur center (shown in *orange* and *yellow spheres*), and the heme *c* group (shown in *red sticks*) of the cytochrome *c*1 subunit (shown in *pink* in A), to finally reduce cytochrome *c*, is represented by the *red arrows* over the protein surface map

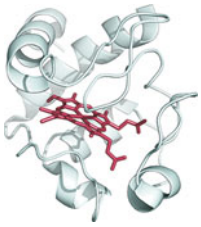


Fig. 6.19 Structure of human cytochrome *c* (PBD 1HCR), showing its heme group in *red sticks*

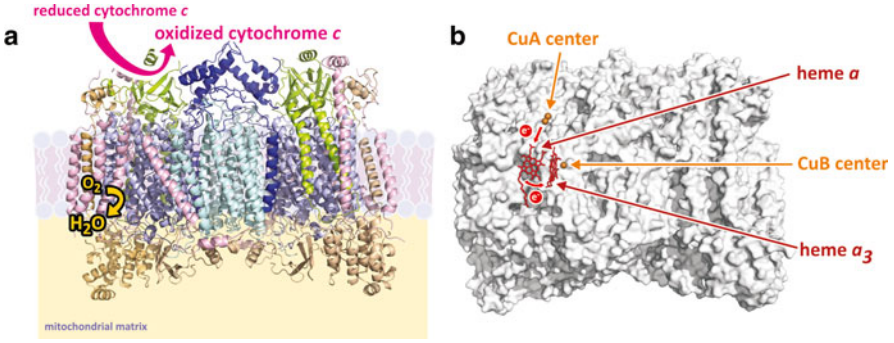


Fig. 6.20 (**a**) Dimeric structure of the bovine cytochrome *c* oxidase (PDB 1OCC), with the 13 subunits of each monomer colored differently. The enzyme catalyzes the oxidation of cytochrome *c* with the reduction of O_2 to H_2O. (**b**) The electrons (e^-) are sequentially transferred through the CuA center (shown in *brown spheres*), to heme *a* (shown in *red sticks*), and heme *a3* (shown in *red sticks*)-CuB center (shown in *brown spheres*) to finally reduce the O_2 to H_2O, as represented by the *red arrows* over the protein surface map

6.2.4 The ATP Synthesis Through Oxidative Phosphorylation

The synthesis of ATP is catalyzed by a large protein complex, the ATP synthase, located, as the respiratory complexes, in the inner mitochondrial membrane. The enzyme uses as substrates ADP and Pi in a reaction dependent on the flow of H^+ from the intermembrane space to the mitochondrial matrix.

In electron microscopy imaging, ATP synthase is seen as characteristic head connected to the membrane by a long stalk. Through studies using the technique of electron cryotomography, it was possible to observe that the ATP synthase dimers are arranged in long rows along the highly curved mitochondrial cristae edges, an organization similar in mitochondria from mammals, fungi, or plants (Fig. 6.21).

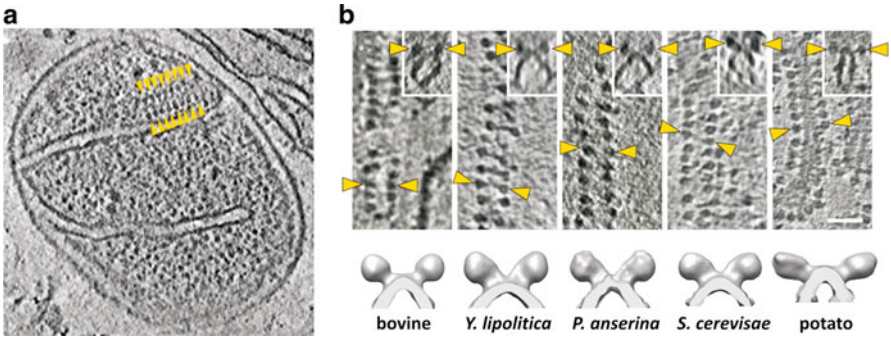


Fig. 6.21 (**a**) Tomographic slices showing the arrays of F_1–F_o ATP synthase dimers (*yellow arrows*) in whole mitochondria of *Podospora anserina*. (**b**) Rows of F_1–F_o ATP synthase in mitochondrial membranes from bovine heart, *Yarrowia lipolytica*, *Podospora anserina*, *Saccharomyces cerevisiae*, and potato. A side view of each array with the dimers in relation to the membrane is shown in the inset. *Yellow arrowheads* indicate F1 heads of one dimer. Scale bar, 50 nm. The surface representation of each dimer is shown in the *bottom*. Figures reproduced with permission from Davies et al. Proc. Natl. Acad. Sci. USA 108:14121–14126, 2011

6.2.4.1 The Structure of ATP Synthase

The overall structure of this large enzyme may be separated in two components: the F_1 portion, a peripheral membrane protein clearly seen in electron microscopy images as projections in the inner mitochondrial membrane (see Fig. 6.21), and the F_o portion (whose denomination comes from its sensitivity to oligomycin), an integral mitochondrial membrane protein, through which H^+ flow from the intermembrane space to the mitochondrial matrix (Fig. 6.22).

The F_1 portion of the ATP synthase was isolated and purified by Efraim Racker, whose studies were decisive for the comprehension of the mechanism of ATP synthesis reaction. The crystallographic structure of F_1, determined by John E. Walker (Fig. 6.22a), revealed that it has nine subunits of five different types, three α-subunits, three β-subunits, and one of each γ-, δ-, and ε-subunits (Fig. 6.22). F_1 δ- and ε-subunits interact with the membrane-embedded transmembrane helices of F_o portion of the enzyme. The catalytic sites of ATP synthesis are located in each of the β-subunits, whose conformations in the enzyme structure are different from each other due to differences in their interactions with the other subunits of the enzyme. This is essential for the mechanism of ATP synthesis, as it will be detailed in the next section.

The F_o complex is composed of three types of subunits: one a subunits; two b subunits, which associate to F_1 α- and β-subunits; and 10–12 small c subunits, which are hydrophobic polypeptides consisting of two transmembrane helices that form a membrane-embedded cylinder that interacts with the δ- and ε-subunits of F_1 complex (see schematic representation in Fig. 6.22b).

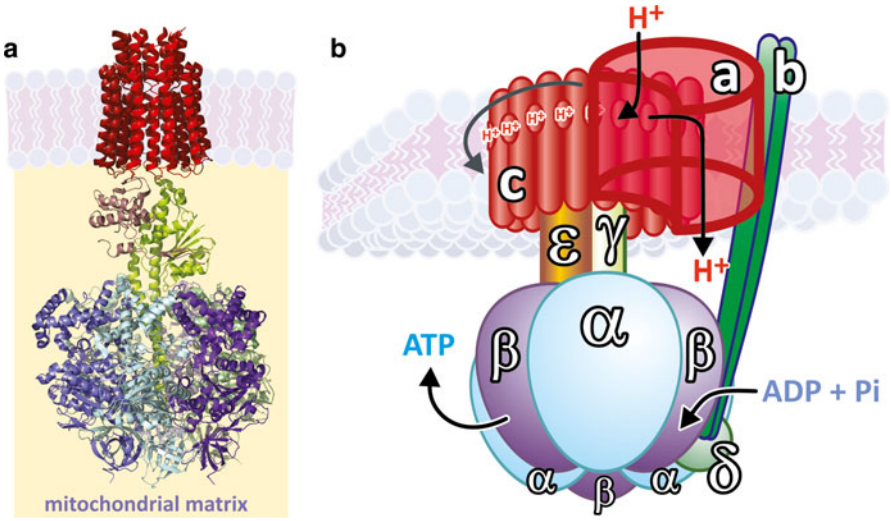


Fig. 6.22 (**A**) Structure of the F_1 and part of the F_o portions of ATP synthase from *Saccharomyces cerevisiae* (PDB 2XOK). The F_1 α-, β-, γ-, and ε-subunits are represented in *cyan*, *purple*, *light green*, and *pink*, respectively. The c subunits of the F_o portions are shown in *red*. (**b**) Schematic representation of the entire F_1F_o-ATP synthase, showing the δ-subunit of F_1 portion as well as the a- and b-subunits of F_o portion, which were not yet determined and thus are not represented in the crystallographic structure shown in (**a**)

6.2.4.2 The Mechanism of ATP Synthesis by the ATP Synthase

The synthesis of ATP from ADP and Pi is a very endergonic reaction in aqueous solution. However, one important point to understand the mechanism of ATP synthesis by ATP synthase is that when occurring in F_1 environment, it is readily reversible, with a free-energy change close to zero (see Box 6.2). For the reaction catalyzed by F_1, the energy barrier consists in the step of ATP release from the enzyme. This energy barrier is overcome by the energy input from the H^+ gradient, since flow through F_o promotes conformational changes in the β-subunit, leading to the loss of its affinity to ATP.

This view of ATP synthesis was formulated by Paul D. Boyer. From his kinetic studies, two main new concepts emerged. The first was that the three catalytic sites of the ATP synthase participate sequentially and cooperatively in the catalytic cycle; the second was that the catalytic mechanism would be seen as "a rotational catalysis" mechanism, as Boyer named, in which the three catalytic sites alternate the reaction catalysis (see Box 6.6). One clue for this proposal was given by the crystallographic structure of F_1, which revealed that the three β-subunits were differentially occupied during the catalytic cycle, one having ADP bound, other having ATP bound, and the third being empty (see Fig. 6.23a).

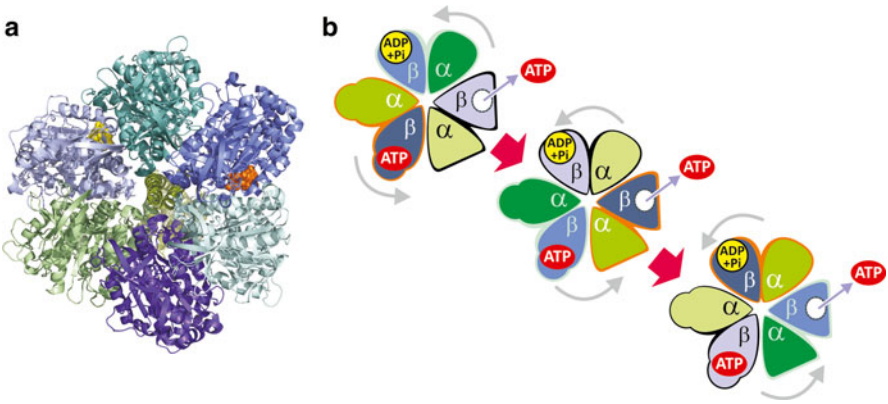


Fig. 6.23 (**a**) Structure of mitochondrial bovine F_1 showing ADP bound to one of the β-subunits, in *yellow*, and a non-hydrolyzable ATP analogue (phosphoaminophosphonic acid-adenylate ester) bound to another β-subunit, in *orange* (PDB 1BMF). (**b**) Schematic representation of the different conformations assumed by F_1 subunits: ADP and Pi bind to the catalytic β-subunit, which is in the β-ADP conformation. The enzyme rotation driven by H^+ flow through F_o portion promotes a conformation change in the β-subunit that acquires the β-ATP conformation, which stabilizes ATP in the active site. Then, another F_1 rotation leads the subunit to its empty conformation, which loses the affinity to ATP, releasing it to the medium

The rotational catalysis mechanism may be summarized in the model shown in Fig. 6.23b. ADP and Pi from the medium bind to the β-subunit catalytic site that is in the β-ADP conformation. The conformation of this β-subunit changes to the β-ATP conformation due to enzyme rotation driven by H^+ flow through F_o portion. In this conformation, the β-subunit stabilizes ATP, which is in equilibrium in the active site with ADP and Pi. Then, another F_1 rotation occurs, leading this β-subunit to change conformation again, now to the empty conformation, which loses the affinity to ATP, releasing it to the medium. Another round starts when another F_1 rotation leads the β-subunit again to the β-ADP conformation. This rotational movement frequently justifies the label of "molecular machine" to ATP synthase.

Box 6.6: The Confirmation of Boyer's Model by Real-Time Microscopy

F_1 rotation could be directly seen in an ingenious experiment performed by the research groups of Masasuke Yoshida and Kazuhiko Kinosita (published in Nature 386:299–302, 1977), in which they attached to the γ-subunit of F_o a long fluorescent actin filament and observed its movement as ATP was hydrolyzed, in real time in a microscope, in relation to the $\alpha_3\beta_3$ core immobilized in the microscope slide. They also observed that the rotation occurred in three discrete steps of 120°, completely confirming Boyer's model.

Due to his great contribution to the understanding of the mechanism of ATP synthesis, Boyer shared the Nobel Prize in Chemistry, in 1997, with John Walker, who determined the crystallographic structure of the F_1 portion of the enzyme, an essential step for the comprehension of the catalytic mechanism.

6.2.5 *Regulation of Oxidative Phosphorylation*

Oxidative phosphorylation is generally limited by the availability of ADP, so that the major control of ATP synthesis by oxidative phosphorylation is the cellular ATP requirement.

When respiratory substrates are freely available, ATP is synthesized so that the ratio ATP/ADP increases. If the levels of ADP become very low (when ATP synthesis overcomes its utilization in cellular metabolism), but the substrates are still available, the H^+ gradient reaches the maximum level. This prevents electron transport and hence respiration, since the gradient cannot be dissipated through ATP synthase, which cannot work due to the absence of its substrate, ADP. This situation is called state 4 respiration (Fig. 6.24) and illustrates the low permeability of the inner mitochondrial membrane to H^+ and the coupling of electron transport to ATP synthesis.

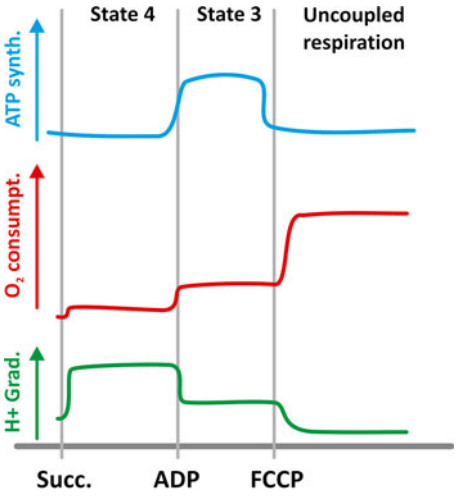


Fig. 6.24 Representation of the extent of the proton gradient, oxygen consumption, and ATP synthesis when a respiratory substrate (e.g., succinate), ADP, and an uncoupler (e.g., *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone, FCCP) are added to intact mitochondria in vitro

When ADP becomes available, the ATP synthase phosphorylates it to ATP, and H^+ gradient is reduced to such an extent that respiration is allowed to proceed, while ADP is available. In this situation, respiration occurs at the same rate of ATP synthesis, in what is called state 3 respiration (Fig. 6.24).

However, even in state 4, or when ADP phosphorylation is inhibited (e.g., using oligomycin), some oxygen consumption is observed, demonstrating that the coupling of respiration to ATP synthesis is imperfect, and part of the energy will be normally dissipated as heat. This occurs to some extent due to H^+ leaking through the inner mitochondrial membrane. This phenomenon can be reproduced by the use of substances called uncouplers (e.g., *p*-trifluoromethoxycarbonylcyanide phenylhydrazone—FCCP), whose effect on O_2 consumption, H^+ gradient, and ATP synthesis is shown in Fig. 6.24. In the presence of uncouplers, H^+ readily move back into the matrix, bypassing the ATP synthase and collapsing the gradient, which causes respiration to be accelerated but uncoupled from ADP phosphorylation.

6.2.5.1 Uncoupling Proteins: The Physiological Uncouplers

Physiologically, uncoupling of electron transport to ADP phosphorylation is provided by a family of uncoupling proteins (UCP). The best studied of them, and the first to be identified, UCP1, is expressed exclusively in a specialized tissue called brown adipose tissue (BAT). In contrast to white adipocytes, brown adipocytes contain several lipid droplets and a much higher number of mitochondria (Fig. 6.25a), which confer the brown color to the tissue.

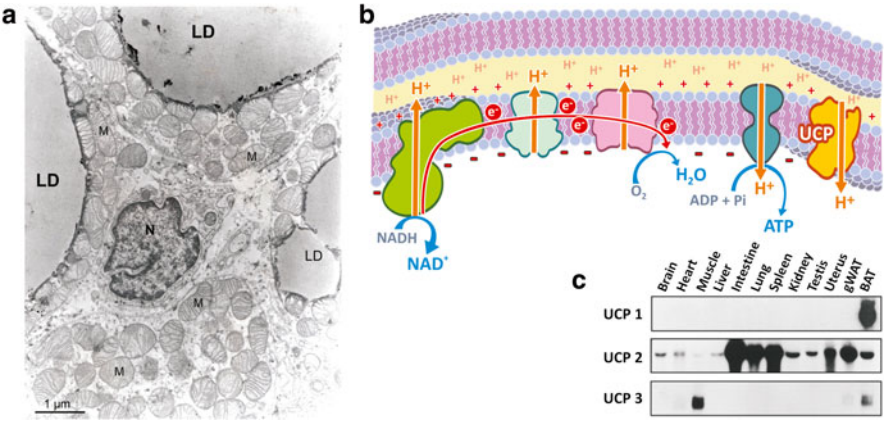


Fig. 6.25 (**a**) Transmission electron micrograph of a BAT adipocyte thin section showing lipid droplets (LD) and a high number of mitochondria (M); N: nucleus. (Courtesy from Prof. Marlene Benchimol). (**b**) The UCPs are integral inner mitochondrial membrane proteins that allow H^+ flow bypassing ATP synthase, dissipating the gradient and accelerating respiration. (**c**) Western blotting showing the expression pattern of the three UCP isoforms in mouse tissues (Reproduced with permission of Portland Press from Ricquier & Bouillaud. Biochem J. 345:161–179, 2000)

In humans, BAT is mostly found in the newborn, regulating thermogenesis through the expression of the UCP1. This protein occurs in abundance in the inner mitochondrial membrane and provides an alternative route, bypassing ATP synthase, for H^+ to return to the mitochondrial matrix, dissipating the gradient and accelerating respiration, resulting in heat production in a regulated manner (Fig. 6.25b). Until very recently, this tissue has been considered to have no physiological relevance to adult humans, but some recent findings suggest that reminiscent BAT cells may proliferate in response to cold exposure (Box 6.7). This effect seems to be more pronounced in lean subjects, suggesting that regulated uncoupled respiration may also be a way to control energy expenditure (see Chap. 11).

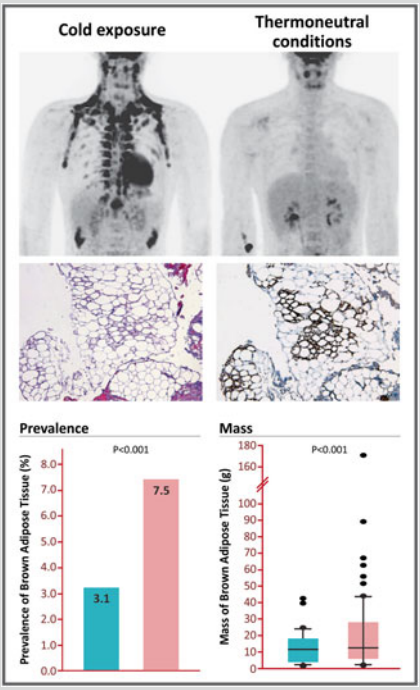
Two other UCP isoforms have been characterized, UCP2 and UCP3. They show a more ubiquitous tissue distribution (Fig. 6.25c) and probably play a role in protecting cells against reactive oxygen species, which may be produced in excess in mitochondria in some situations, such as when an excessive amount of substrates is supplied to the cells (see next section).

Box 6.7: BAT in Adult Humans

Positron-emission tomography combined with computed tomographic (PET–CT) scans, with ^{18}F-fluorodeoxyglucose (^{18}F-FDG) as a tracer, is generally used to diagnose neoplasms and their metastases, since tumor cells present a much higher glucose uptake when compared to other cells (see Box 6.4 about the Warburg effect). In these tests, a high glucose uptake in the supraclavicular tissue is usually observed, sometimes confusing the diagnosis. It was speculated that these highly glycolytic cells in this region would be brown adipose tissue (BAT). Recently, a set of studies was performed to investigate this issue. In some of these studies, healthy volunteers were exposed to 16 °C for 2 h before the tests. Comparative PET–CT scans revealed a great increase in ^{18}F-FDG uptake in the supraclavicular region upon cold exposure (see figure). Furthermore, tissue biopsies were used for immunostaining with UCP1-specific antiserum, confirming the presence of substantial amounts of metabolically active BAT in adult humans. A systematic examination of the presence and distribution of BAT in lean and obese men during exposure to cold temperature showed that BAT activity is inversely correlated to body mass index (BMI). Additionally, analysis of 3640 consecutive PET–CT scans performed for various diagnostic reasons in 1972 patients showed substantial BAT depots in regions extending from the anterior neck to the thorax for 76 of 1013 women (7.5 %) and 30 of 959 men (3.1 %), with also a larger mass in women.

(continued)

Box 6.7 (continued)



Presence of BAT in humans detected by PET-CT scans after exposure to cold (*top left*) and under thermoneutral conditions (*top right*), and histologic images of biopsy specimens showing UCP1 staining (*middle right*), and the quantification of prevalence (*bottom left*) and amount (*bottom right*) of BAT in men (*blue*) and women (*pink*). (Reproduced with permission from Lichtenbelt et al. New Engl. J. Med. 360:1500–1508, 2009, and Cypess et al. New Engl. J. Med. 360:1509–1517, 2009)

6.2.5.2 Production of Reactive Oxygen Species in Mitochondria

In physiological or pathological situations in which the input of electrons into the respiratory chain overcomes their transfer to oxygen, such as in hypoxia, the formation of reactive species of oxygen (ROS) is increased. This occurs because the passage of electrons from complex I to ubiquinone and from ubiquinone to complex III involves the formation of a partially reduced ubiquinone radical ($^{\bullet}Q^{-}$) as an intermediate. When the electron flow through the respiratory chain is impaired, the probability of this radical to react with cellular components before being completely reduced or oxidized greatly increases. $^{\bullet}Q^{-}$ can react with oxygen, generating the superoxide radical ($^{\bullet}O_2^{-}$). This radical is very reactive and its formation may lead to the production of an even more reactive radical, the hydroxyl radical ($^{\bullet}OH$). These

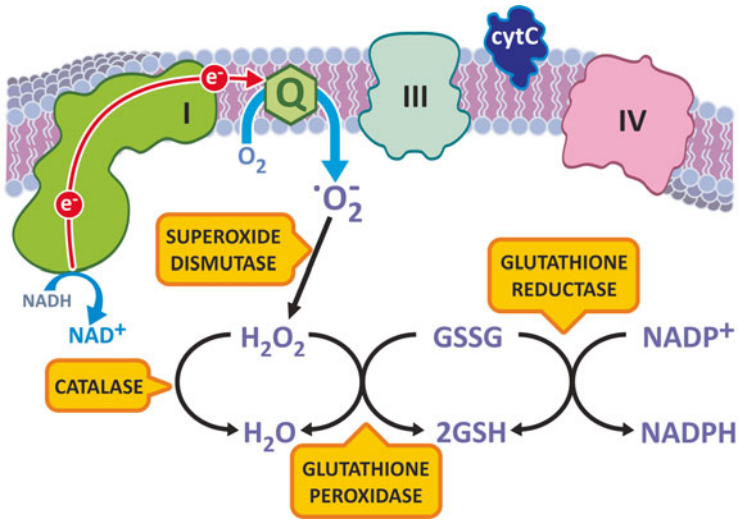


Fig. 6.26 A partially reduced ubiquinone radical ($^{\bullet}Q^{-}$) is formed as an intermediate in the reduction of ubiquinone to ubiquinol by complex I or the oxidation of ubiquinol to ubiquinone by complex III. If $^{\bullet}Q^{-}$ accumulates, it may react with O_2, forming $^{\bullet}O_2^{-}$ and $^{\bullet}OH$. Cells have different enzymatic systems to prevent oxidative damage caused by ROS, including the enzymes superoxide dismutase, glutathione peroxidase and catalase

ROS can react and damage enzymes, lipids, and nucleic acids. They can also alter cellular gene expression, leading to several modifications in cellular functions.

Cells have different enzymatic systems to prevent oxidative damage caused by ROS. This includes the enzyme superoxide dismutase that converts $^{\bullet}O_2^{-}$ in hydrogen peroxide (H_2O_2), which in turn may be used by glutathione peroxidase to reduce glutathione or by catalase to form H_2O (Fig. 6.26).

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