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Review

Intrinsic and extrinsic uncoupling of oxidative phosphorylation ☆

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Abstract

This article reviews parameters of extrinsic uncoupling of oxidative phosphorylation (OxPhos) in mitochondria, based on induction of a proton leak across the inner membrane. The effects of classical uncouplers, fatty acids, uncoupling proteins (UCP1–UCP5) and thyroid hormones on the efficiency of OxPhos are described. Furthermore, the present knowledge on intrinsic uncoupling of cytochrome *c* oxidase (decrease of H^+/e^- stoichiometry = slip) is reviewed. Among the three proton pumps of the respiratory chain of mitochondria and bacteria, only cytochrome *c* oxidase is known to exhibit a slip of proton pumping. Intrinsic uncoupling was shown after chemical modification, by site-directed mutagenesis of the bacterial enzyme, at high membrane potential $\Delta\Psi$, and in a tissue-specific manner to increase thermogenesis in heart and skeletal muscle by high ATP/ADP ratios, and in non-skeletal muscle tissues by palmitate. In addition, two mechanisms of respiratory control are described. The first occurs through the membrane potential $\Delta\Psi$ and maintains high $\Delta\Psi$ values (150–200 mV). The second occurs only in mitochondria, is suggested to keep $\Delta\Psi$ at low levels (100–150 mV) through the potential dependence of the ATP synthase and the allosteric ATP inhibition of cytochrome *c* oxidase at high ATP/ADP ratios, and is reversibly switched on by cAMP-dependent phosphorylation. Finally, the regulation of $\Delta\Psi$ and the production of reactive oxygen species (ROS) in mitochondria at high $\Delta\Psi$ values (150–200 mV) are discussed.

Keywords: Uncoupling of oxidative phosphorylation; Proton pumping; Membrane potential; Cytochrome *c* oxidase; ROS production; Thyroid hormone; Protein phosphorylation; ATP synthase

1. Introduction

Aerobic organisms synthesize ATP mainly by two routes, (1) by glycolysis in the cytosol, and (2) by oxidative phosphorylation (OxPhos) on membranes; on the plasma membrane in bacteria, or on the inner mitochondrial membrane in eukaryotes. With glucose as substrate, OxPhos yields about 17 times more ATP than glycolysis. Therefore, OxPhos represents the main source of ATP in aerobic organisms.

OxPhos comprises a respiratory chain with three proton pumps (NADH dehydrogenase = complex I, cytochrome *c* reductase or cytochrome bc_1 complex = complex III, and cytochrome *c* oxidase = complex IV), an enzyme complex not pumping protons (succinate dehydrogenase = complex II), and the ATP synthase (complex V), a fourth reversible proton pump, using the energy of the proton gradient across the membrane to synthesize ATP [1–6]. Crystal structures are available for complex II (fumarate reductase) from *Wolinella succinogenes* [7] and *Escherichia coli* [8], for complex III from yeast [9,10] and bovine heart [11–13], for complex IV from *Paracoccus denitrificans* [14,15] and bovine heart [16–18], and for the F_1 part of complex V (ATP synthase) from bovine heart [1] and rat liver [2]. According to Peter Mitchell's chemiosmotic theory, the energy-rich intermediate of OxPhos is the proton gradient across the membrane $\Delta\mu_{H^+}$. Its driving force was defined by Mitchell [19,20] as the proton motive force Δp ($\Delta p = \Delta\mu_{H^+}/F$, F = Faraday constant), consisting of an electrical ($\Delta\Psi_m$) and a chemical part (ΔpH_m) [21]:

$$\Delta p = \Delta\Psi - 59\Delta pH \ [\mathrm{mV}]$$

The yield of OxPhos was for long time a matter of discussion [22] and turned out to be variable (see below). A scheme of OxPhos in mitochondria is presented in Fig. 1.

☆ Dedicated to my old friend Professor Giovanni Felice Azzone, who introduced the terms intrinsic and extrinsic uncoupling of oxidative phosphorylation.

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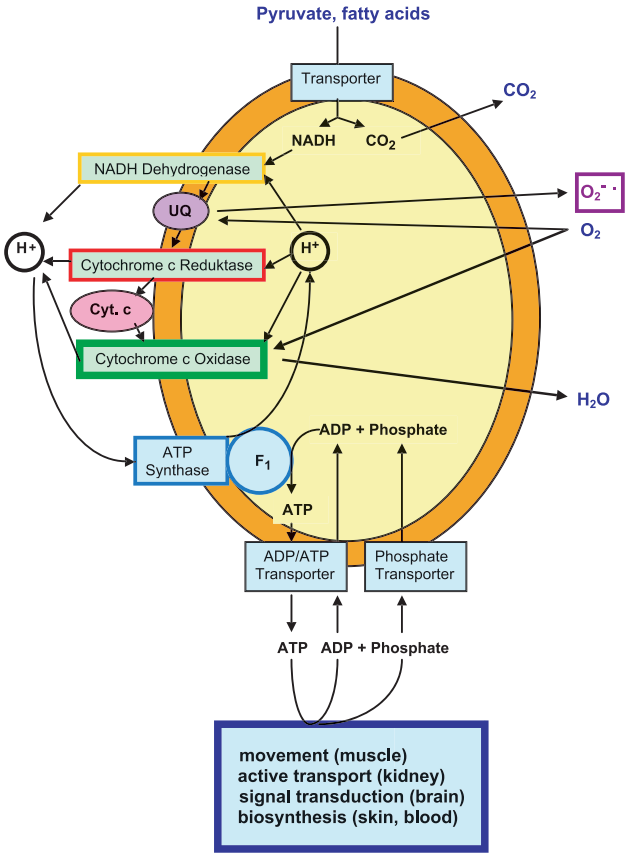

Fig. 1. Scheme of OxPhos in mitochondria. The basic principle of OxPhos, the outward translocation of protons by complexes I (NADH dehydrogenase), III (cytochrome *c* reductase) and IV (cytochrome *c* oxidase) and the uptake of the protons by the ATP synthase, accompanied by the synthesis of ATP, is shown. The formation of the superoxide anion radical (O_2^-) by the transfer of one electron from ubiquinone to dioxygen is also indicated.

The multiple subunits of the four proton pumps of OxPhos in animals are partly encoded on nuclear DNA and partly on mitochondrial DNA (mtDNA). The 13 mtDNA-encoded protein subunits (1–7 in each complex) represent the only proteins encoded on the circular mtDNA molecule (16 569 bp in human). Thus, in animals, OxPhos is controlled by the mitochondrial genome, which, in contrast to the nuclear genome, is only inherited by the mother [23]. It is generally accepted that mtDNA plays a major role in aging, due to slow decrease of the capacity of OxPhos in mitochondria via stochastic somatic mutations of mtDNA [24–26].

The stoichiometric efficiency of OxPhos is defined by the P/O ratio, or the amount of inorganic phosphate (Pi) incorporated into ATP per amount of consumed oxygen. Uncoupling of OxPhos describes any process which decreases the P/O ratio, mostly based on decrease of Δp, thus leading to waste of redox energy and increased thermogenesis. Multiple parameters could degrade the proton gradient across the membrane and decrease the P/O ratio. These include classical uncouplers of OxPhos [27,28], fatty acids [29], an unspecific "proton leak" of the inner mitochondrial membrane [28], and the uncoupling protein (UCP1) of brown adipose tissue [30–34]. In addition, any active transport of cations or anions across the membrane via transporter (carrier) will decrease Δp. In mitochondria, the uptake of Ca^{2+} by the calcium carrier (uniport) [35], and of ADP^{3-} in exchange with ATP^{4-} by the ADP carrier (antiport) [36], decrease $\Delta\Psi_m$ (electrogenic transport). The electroneutral uptake of negatively charged phosphate together with a H^+ by the phosphate carrier (symport) [37] decreases ΔpH, and the exchange of mitochondrial aspartate against glutamate and a H^+ by the aspartate/glutamate carrier [38] decreases Δp. The metabolite and cation carriers of mitochondria, however, will not be subject of this review.

In contrast to the above components, which degrade the proton gradient ("extrinsic uncoupling"), a decrease of the efficiency of the proton pumps (i.e. decrease of the H^+/e^- or H^+/ATP stoichiometry) would also result in a diminished P/O ratio ("intrinsic uncoupling" or "slip" of proton pumps). The concept of intrinsic and extrinsic uncoupling was introduced by Azzone et al. [39]. A scheme for intrinsic and extrinsic uncoupling of OxPhos in mitochondria is presented in Fig. 2.

The present article reviews parameters of extrinsic uncoupling and describes the present knowledge on intrinsic uncoupling of cytochrome *c* oxidase, the terminal enzyme of the respiratory chain in mitochondria and some bacteria. Remarkably, only complex IV and possibly complex V, not complex I and III, were shown to exhibit intrinsic uncoupling. In addition, the control of mitochondrial respiration, the regulation of the mitochondrial membrane potential $\Delta\Psi_m$ and the production of reactive oxygen species (ROS) are discussed.

2. Evolution of OxPhos

Life on earth originated in the absence of oxygen. After increasing amounts of dioxygen accumulated in the atmosphere, due to the activity of photosynthetic organisms, an efficient oxidative metabolism evolved which is presently more or less the same in plants, animals and most bacteria. Also the structures of the enzymes of glycolysis and OxPhos are very similar in animals and most bacteria. One remarkable difference, however, is the regulatory complexity of respiratory chain complexes, as manifested in their subunit composition. Complexes I, II, III and IV of mammals contain 42, 4, 11 and 13 subunits, compared to only 13, 4, 3 and 4 in *P. denitrificans*, respectively. Interestingly, only the proton pumps (complexes I, III and IV) contain additional subunits in eukaryotes (19, 8 and 9, respectively).

The physiological significance of additional regulation of the respiratory chain in animals compared to bacteria could be the following. Plants and heterotrophic organisms produce large amounts of organic matter. During some geological periods, this organic matter has been stored in coal,

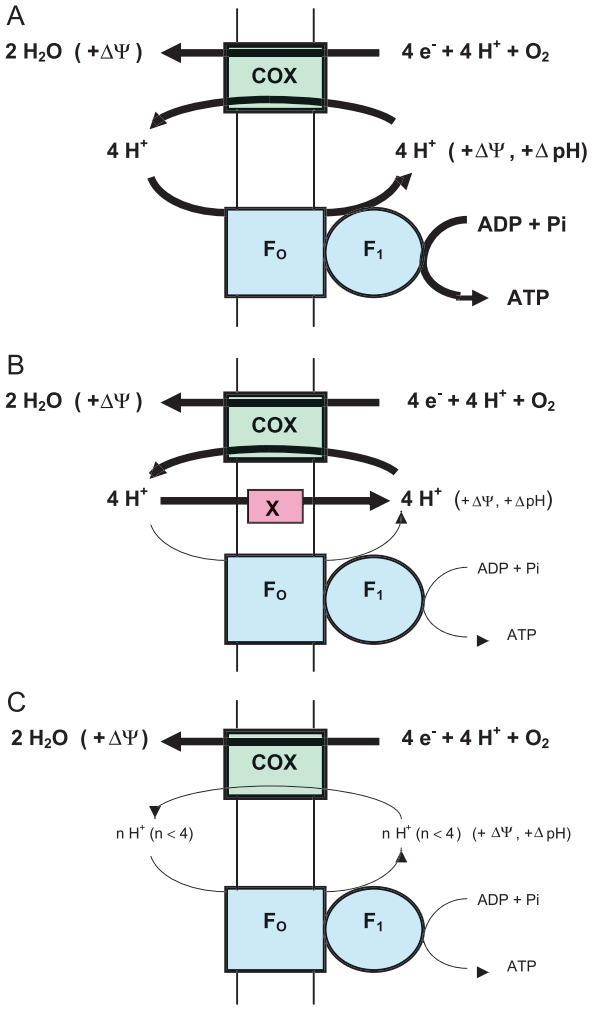

Fig. 2. Simplified scheme of OxPhos (A), of its intrinsic uncoupling (B) via decreased H^+/e^- stoichiometry (slip) in cytochrome *c* oxidase (COX), and of its extrinsic uncoupling (C) by backflow of protons across the membrane (leak) via uncoupler of OxPhos (X). The scheme is limited to the presentation of cytochrome *c* oxidase and the ATP synthase (F_oF_1).

mineral oil and natural gas. At present, however, almost an equilibrium exists between the production and degradation of organic matter on earth. This equilibrium is only stable if the degrading organisms (mostly bacteria) have an excess capacity (activity). Thus, if the environmental conditions are adequate, bacteria will grow until all substrates (organic matter) are used up. In contrast, animals utilize energy (consume substrates) only under selected conditions, and in a tissue-specific manner. Therefore, the synthesis and utilization of ATP in multicellular organisms is complex regulated, involving a variety of hormonal and neuronal signal cascades.

3. Energy metabolism of endotherms

Warm-blooded animals require energy mainly for three purposes: (1) for the maintenance of body structure (e.g. continuous biosynthesis and degradation of constituents); (2) for work activities (e.g. mechanical, electrical, and osmotic work); and (3) for thermogenesis (maintenance of body temperature). Thermogenesis consumes a large part of the total energy produced in endotherms, and a 7-fold higher resting metabolism was determined in rats compared with the reptile *Amphibolurus vitticeps* of comparable weight [40]. Energy consumed by work and thermogenesis varies largely under different conditions. Therefore, OxPhos has to be regulated mainly in two respects: (1) regulation of the rate of ATP synthesis and thus the rate of respiration, and (2) regulation of the efficiency of ATP synthesis and thus the extent of thermogenesis.

3.1. Regulation of the rate of OxPhos

The rate of ATP synthesis according to its utilization is controlled by a feedback mechanism named ‘‘respiratory control’’ (see Section 4). Other mechanisms regulate the efficiency and maximal rates of OxPhos.

According to classical equilibrium thermodynamics, maximal efficiency of energy converters is only obtained when the energy-transducing processes are fully coupled and the system is in a state of thermodynamic equilibrium. For biological systems, this condition does not hold, because at thermodynamic equilibrium, all net flows and thermodynamic forces vanish. To obtain the necessary flow rate, the reactions have to proceed far away from thermodynamic equilibrium (at flow equilibrium). For the quantitative description of efficiency under these conditions, Kedem and Kaplan [41] applied the formalism of nonequilibrium thermodynamics. They introduced the term coupling degree *q* as a measure of the efficiency of an irreversible reaction, which can have values between 0 and 1.

Stucki [42] calculated the coupling degrees *q* for ATP synthesis in mitochondria under various conditions. For a maximal rate of ATP synthesis, a *q* value of 0.786 was calculated. For maximal ATP synthesis at optimal efficiency, a higher *q* value of 0.91 was obtained, and for maximal ATP synthesis and optimal efficiency, under most economic conditions, a coupling degree of $q = 0.97$ was calculated.

A variable degree of coupling between the ‘‘proton motive force’’ Δp and the rate of respiration, indicating variable efficiency, was first described by Nicholls [43] with rat liver mitochondria respiring with succinate. He observed a non-ohmic behaviour of the mitochondrial membrane and explained it by proton leak (unspecific proton permeability of membranes at high Δp), but did not exclude slip of proton pumps (decreased coupling between electron transport and proton translocation). Pietrobon et al. [44,45] and Zoratti et al. [46] postulated a slip at high Δp in the mitochondrial respiratory chain, in particular in cytochrome *c* oxidase [39]. Recent results with the isolated enzyme corroborated the slip in cytochrome *c* oxidase, and suggest tissue-specific regulation of the efficiency and thus of the rate of OxPhos (see Section 7).

3.2. Regulation of the efficiency of OxPhos

One way of changing the efficiency of OxPhos is by delivering the reducing equivalents of substrates into the mitochondrial respiratory chain at different steps. Aerobic oxidation of cytosolic NADH involves either import of reducing equivalents into mitochondria by the malate–aspartate shuttle or by the glycerophosphate shuttle. The glycerophosphate shuttle omits the first phosphorylation step at complex I, yielding lower P/O ratios (decreased efficiency), accompanied by faster rates of respiration and increased thermogenesis. An increased rate of cytosolic NADH oxidation, and thus of glycolytic ATP synthesis, is important in skeletal muscle at high energy expenditure. Therefore, in skeletal muscle, high levels of the mitochondrial FAD glycerol-1-phosphate dehydrogenase are expressed. Its activity becomes most effective during exercise when increased calcium levels occur, because the enzyme is activated by calcium [47]. Thyroid hormones increase the glycerophosphate shuttle by increasing the mitochondrial enzyme level, in particular, in liver and heart [48].

Other ways of decreasing the efficiency of OxPhos are described below and concern the production (slip = intrinsic uncoupling) or degradation (leak = extrinsic uncoupling) of the proton motive force Δp (in mitochondria mainly $\Delta\Psi_m$).

3.3. Regulation of OxPhos through cytochrome c oxidase

The function of additional subunits in respiratory chain complexes from eukaryotes as compared to those from prokaryotes was for long time a mystery. In fact, the same catalytic properties were found with cytochrome *c* oxidase from *P. denitrificans* (2–3 subunits) and from bovine heart (13 subunits) [49,50]. For the "supernumerary" subunits, structural roles or assistance in assembly were suggested, but also functions in catalysis were proposed [51,52], and lately verified by experiments [53–56]. Nevertheless, in a recently published book on bioenergetics [57], the authors state for cytochrome *c* oxidase: "The key catalytic functions are found in each case in subunits I and II (Plate C). Other subunits (as many as 11 for the mitochondrial enzyme) are not relevant to catalysis and are not considered here". As will be shown below, the supernumerary subunits IV (Section 4.2), Va (Section 4.3) and VIa (Section 7.2.4) of mammalian cytochrome *c* oxidase are involved in regulation of the rate of respiration as well as efficiency of proton translocation, thus having a profound influence on OxPhos. Regulatory functions of other nuclear coded subunits of mammalian cytochrome *c* oxidase, which do not occur in prokaryotes, may be inferred from the identification of seven high-affinity binding sites for ATP or ADP, and three additional only for ADP, at the bovine heart enzyme [58,59]. Furthermore, reversible phosphorylation of subunits was shown (see Section 4.4.2); and with antibodies against phosphoserine and phosphothreonine, at least 8 of the 13 subunits revealed immunological reactivities at variable extent, depending on the isolation conditions of the bovine heart enzyme [60].

4. Control of mitochondrial respiration

Living cells are characterized by homeostasis of metabolites. This holds in particular for the components of the energy charge [ATP]/[ADP][Pi]. The mechanism which adapts the rate of respiration—in general corresponding to the rate of ATP synthesis by OxPhos—to the rate of ATP utilization is named "respiratory control".

4.1. First mechanism of respiratory control

"Respiratory control" was originally defined as stimulation of the respiration of isolated mitochondria (oxygen consumption) by ADP (active, state 3 respiration), followed by its decrease after conversion of ADP into ATP (controlled, state 4 respiration) [61,62]. This phenomenon was explained by the chemiosmotic theory [19,20]: Uptake of ADP into mitochondria stimulates the ATP synthase accompanied by a decrease of Δp, which in consequence stimulates the activity of the three proton pumps and thus mitochondrial respiration [21]. The proton pumps are inhibited at high $\Delta\Psi_m$ values (150–200 mV). The chemiosmotic theory was strongly supported by the effect of "uncouplers" of OxPhos which increase the proton conductance of biological membranes (see Section 6.1), dissipate Δp and result in increased respiration, not coupled to phosphorylation.

4.2. Second mechanism of respiratory control

A "second mechanism of respiratory control" was discovered based on the allosteric ATP inhibition of cytochrome *c* oxidase at high intramitochondrial ATP/ADP ratios [54,63]. ATP converts the hyperbolic into sigmoidal inhibition kinetics with a Hill coefficient of 2, suggesting induction of cooperativity by ATP between the two binding sites for cytochrome *c* within the dimeric enzyme complex [17]. ATP or ADP bind to the same binding site at the matrix domain of the transmembrane subunit IV, representing one of seven high-affinity binding sites for ATP or ADP in the bovine heart enzyme [58,59]. The binding site at subunit IV was concluded from prevention of ATP inhibition after preincubation of the enzyme with a monoclonal antibody against subunit IV [54]. The second mechanism of respiratory control is independent of Δp and occurs to half-maximal extent at an intramitochondrial ATP/ADP ratio of 28 [64], which lies in the physiologically expected range (see Lee et al. [65]). The allosteric ATP inhibition of cytochrome *c* oxidase does not occur in the bacterial enzyme [66], and is reversibly switched on and off by hormones (see below). The second mechanism of respiratory control is suggested to maintain in vivo low $\Delta\Psi_m$

values (100–150 mV), due to the potential dependence of the ATP synthase (see Section 5.1). The various physiological and unphysiological parameters which switch off the allosteric ATP inhibition of cytochrome *c* oxidase are listed in Table 1.

4.3. Regulation by thyroid hormones

Thyroid hormones represent the major regulator of the basal metabolic rate (BMR) in mammals. They increase energy expenditure partly by reducing metabolic efficiency [67]. Thyroid hormones act in two ways; binding of 3,5,5′ -triiodo-L-thyronine (T3) to the thyroid hormone receptor stimulates or inhibits the expression of specific genes [68], resulting in long-term (1–3 days) increase of catabolic and decrease of anabolic enzymes [69]. The short-term effects of thyroid hormones are based on direct interaction of 3,5-diiodo-L-thyronine (T2) with enzymes [67,70], and result in immediate increase of the BMR [71,72]. T2 is formed intracellularly from T3 by deiodination [73]. T2 binds to subunit Va of cytochrome *c* oxidase, as shown by using the radioactive hormone ([3-^{125}I],5-diiodothyronine) [55]. The binding abolishes the allosteric ATP inhibition of cytochrome *c* oxidase with half-maximal effects at 10^{-7} M. The abolition of allosteric ATP inhibition is specific for T2 and prevented by a monoclonal antibody against subunit Va. The effect of T2 on cytochrome *c* oxidase could result in partial uncoupling of OxPhos via increased $\Delta\Psi_m$ (due to relief of the second mechanism of respiratory control) and decreased H^+/e^- stoichiometry (intrinsic uncoupling) of cytochrome *c* oxidase at higher membrane potentials (Section 7.2.3), and thus in increased BMR. This interpretation is supported by the observation that the increase of BMR by T2 is strictly correlated with increased expression of the uncoupling proteins UCP2 or UCP3, which are suggested to prevent ROS formation at high $\Delta\Psi_m$ (see Section 6.3).

Table 1
Parameters which abolish the allosteric ATP inhibition of mammalian cytochrome *c* oxidase

Unphysiological parameters
(1) Use of dodecylmaltoside as solubilizing detergent [54]
(2) Use of TMPD for measurement of enzyme activity [54]
(3) Insufficient cardiolipin in the isolated or reconstituted enzyme [54]
(4) High concentrations of ethanol (above 40 mM) (S. Hammerschmidt and B. Kadenbach, unpublished data)

Physiological parameters
(1) 3,5-Diiodo-L-thyronine via binding to subunit Va (half-maximal effect at 0.1 μM) [55]
(2) High substrate concentrations (high ferro-/ferricytochrome *c* ratio) due to the sigmoidal *v*/*S* relationship [54,64]
(3) Ca^{2+}-activated dephosphorylation of cytochrome *c* oxidase [80] (possibly at Ser^{441} of subunit I of the bovine enzyme [65])
(4) Submicromolar concentrations of palmitate (half-maximal effect at 0.3 μM) (S. Hammerschmidt and B. Kadenbach, unpublished data)

4.4. Regulation via reversible phosphorylation

The supply of reducing equivalents to the respiratory chain (the NADH/NAD^+ ratio) is partly regulated by reversible phosphorylation of the pyruvate dehydrogenase complex via calcium. Phosphorylation inactivates the enzyme complex, whereas dephosphorylation by a calcium-activated protein phosphatase activates it [74,75]. In mitochondria, specific protein kinases and protein phosphatases have been described [76,77], but only recently, defined target proteins of the respiratory chain, namely, subunits of NADH dehydrogenase and cytochrome *c* oxidase, could be identified.

4.4.1. Phosphorylation of NADH dehydrogenase

A cAMP-dependent phosphorylation of the 18-kDa protein subunit of mammalian NADH dehydrogenase was described by Scacco et al. [78] and Papa et al. [79]. Phosphorylation stimulates the activity of complex I and the respiratory activity of myoblast cultures with NAD-linked substrates. Mutations in the gene for the 18-kDa protein (NDUFS4) result in deficiency of complex I and fatal neurological syndromes. The 18-kDa protein is phosphorylated by a cAMP-dependent protein kinase in the matrix, and dephosphorylated by a Ca^{2+}-inhibited protein phosphatase.

4.4.2. Phosphorylation of cytochrome c oxidase

The allosteric ATP inhibition of cytochrome *c* oxidase was found to be switched on by cAMP-dependent phosphorylation and switched off by Ca^{2+}-activated dephosphorylation of the enzyme [80]. From radioactive labelling of the isolated bovine heart enzyme with [γ-^{32}P]ATP, from the sidedness of cAMP-dependent labelling, and from the occurrence of consensus sequences for cAMP-dependent phosphorylation, Ser^{441} of subunit I of bovine heart cytochrome *c* oxidase was concluded to represent the site of cAMP-dependent phosphorylation [65]. Ser^{441} is accessible from the cytosolic side and therefore suggested to be phosphorylated by a cAMP-dependent protein kinase and dephosphorylated by a calcium-activated protein phosphatase, both located at the intermembrane space of mitochondria.

The effects of cAMP-dependent phosphorylation and Ca^{2+}-inactivated or -activated dephosphorylation of NADH dehydrogenase and cytochrome *c* oxidase, respectively, are suggested to modify OxPhos in the same direction. cAMP-dependent phosphorylation of NADH dehydrogenase stimulates its activity. cAMP-dependent phosphorylation of cytochrome *c* oxidase optimizes the efficiency of OxPhos by keeping the mitochondrial membrane potential low via the second mechanism of respiratory control. At low [Ca^{2+}], a matrix protein phosphatase could dephosphorylate and inactivate NADH dehydrogenase, the extent of which depends on the activities of both, phosphorylation and dephosphorylation. ‘‘Stress hormones’’ are assumed to increase cytosolic (and matrix) [Ca^{2+}] and prevent dephosphorylation and thus inactivation of NADH dehydrogenase.

In addition, calcium relieves the second mechanism of respiratory control via dephosphorylation of cytochrome *c* oxidase, and thus increases the power of OxPhos by increasing $\Delta\Psi_m$, but simultaneously decreases its efficiency by increased slip (intrinsic uncoupling).

5. Control of the mitochondrial membrane potential $\Delta\Psi_m$

Biological membranes are more or less impermeable to protons at low membrane potentials, but above 140 mV, the proton permeability of phospholipid and mitochondrial membranes increases exponentially [81]. In contrast, a linear relation was found between H^+ flux and the pH gradient. The increased proton flux at $\Delta\Psi_m$>140 mV results in uncoupling of OxPhos. Therefore, a strong control of $\Delta\Psi_m$ in vivo is required. The various parameters which influence $\Delta\Psi_m$ are summarized in Table 2.

5.1. Dependence of ATP synthase activity on membrane potential

In contrast to the previous view, that the two components of the proton motive force, $\Delta\Psi$ and ΔpH are thermodynamically and kinetically equivalent driving forces for the synthesis of ATP by F_0F_1–ATP synthases [82,83], Dimroth et al. and Kaim and Dimroth verified the membrane potential as the essential driving force for rotation of the "rotor" $\gamma\varepsilon c_n$ of the synthase [84,85] (see Section 7.1). The influence of $\Delta\Psi$ on the rates of ATP synthesis by F_0F_1–ATP synthases has been investigated with the reconstituted Na^+-dependent enzyme from *Propionigenium modestum* and with the H^+-dependent enzymes from spinach chloroplasts and *E. coli* [85]. The authors could demonstrate that the chloroplast ATP synthase is not driven by ΔpH alone, based on the original 'acid bath procedure' by Jagendorf and Uribe [86], but requires $\Delta\Psi$. They showed that the previously applied succinate buffer forms in addition to ΔpH also a membrane potential of about 150 mV, which is essential for ATP synthesis at high catalytic rate. Titration of the rates of ATP synthesis against the membrane potential with the enzymes of *E. coli* and *P. modestum* resulted in half-maximal rates of ATP synthesis at about 80 mV with saturation at 100–120 mV. With the chloroplast enzyme, saturation of ATP synthesis was already obtained at 50–60 mV. It is reasonable to assume also for the mammalian ATP synthase saturation and maximal rates of ATP synthesis at $\Delta\Psi$ values of 100–120 mV.

Table 2
Regulation of the mitochondrial membrane potential $\Delta\Psi_m$

Parameters which decrease the mitochondrial membrane potential $\Delta\Psi_m$
(1) Low ATP/ADP ratio, via stimulation of ATP synthase activity
(2) Low NADH/NAD^+ ratio, due to substrate limitation of the respiratory chain
(3) Uncoupler of OxPhos by increasing the proton permeability of the membrane (leak)
(4) Phosphorylation of cytochrome *c* oxidase at subunit I (Ser^{441} of the bovine enzyme), due to switching on the second mechanism of respiratory control [65]
(5) Superoxide radical anion, due to activation of uncoupling proteins [119]

Parameters which increase the mitochondrial membrane potential $\Delta\Psi_m$
(1) High ATP/ADP ratio, due to inhibition of ATP synthase activity
(2) High NADH/NAD^+ ratio, due to high substrate pressure at the respiratory chain
(3) Dephosphorylation of cytochrome *c* oxidase at subunit I (Ser^{441} of the bovine enzyme), due to switching off the second mechanism of respiratory control [65]
(4) 3,5-Diiodo-L-thyronine (half-maximal at 0.1 μM), due to abolition of the second mechanism of respiratory control [55]
(5) Palmitate (half-maximal at 0.3 μM), due to abolition of the second mechanism of respiratory control (S. Hammerschmidt and B. Kadenbach, unpublished data)

Consequences of high mitochondrial membrane potential ($\Delta\Psi_m$>150 mV)
(1) Formation of reactive oxygen species (ROS) [96–98]
(2) Exponential increase of membrane proton permeability (leak) [81]
(3) Slip in cytochrome *c* oxidase (decrease of H^+/e^- stoichiometry) [39,164,182–184]
(4) Decreased efficiency of OxPhos, due to (2) and (3)
(5) Increased thermogenesis, due to (2) and (3)
(6) Maximal rates of OxPhos under high rates of ATP utilization (see Section 5.2)

5.2. Regulation of $\Delta\Psi_m$ by the second mechanism of respiratory control

The potential dependence of the ATP synthase [85] and the inhibition of mitochondrial respiration by high ATP/ADP ratios (interacting with cytochrome *c* oxidase) represent a feedback system which controls respiration according to the utilization of ATP. This feedback system is different from the "first mechanism of respiratory control" [21], and was suggested to keep in vivo the mitochondrial membrane potential $\Delta\Psi_m$ at low values [65], close to optimal values for the synthesis of ATP by the ATP synthase (100–140 mV) [85], as presented in the scheme of Fig. 3. Under stress conditions, an increase of $\Delta\Psi_m$ was suggested, due to calcium-activated dephosphorylation of cytochrome *c* oxidase and control of respiration by $\Delta\Psi_m$, instead by the ATP/ADP ratio. An increase of $\Delta\Psi_m$ as a consequence of increased calcium levels was indeed measured in intact cells. Addition of vasopressin or thapsigargin to cultivated hepatocytes increased cellular [Ca^{2+}] accompanied by increase of $\Delta\Psi_m$ and ΔpH_m [87,88]. Also, induction of apoptosis in human T lymphocytes by the fungicide tributyltin preceded an increase of cytosolic [Ca^{2+}] and hyperpolarization (increase) of $\Delta\Psi_m$, followed by release of cytochrome *c* and decrease of $\Delta\Psi_m$ [89].

In isolated mitochondria [21,90] and with reconstituted cytochrome *c* oxidase [91,92], high membrane potentials of $\Delta\Psi_m$ = 140–200 mV have been measured. These high $\Delta\Psi_m$ values in vitro contrast low values, determined by Wan et al. [93] in the perfused rat heart. At low cardiac work, $\Delta\Psi_m$ values of 118 mV with glucose and 145 mV with pyruvate,

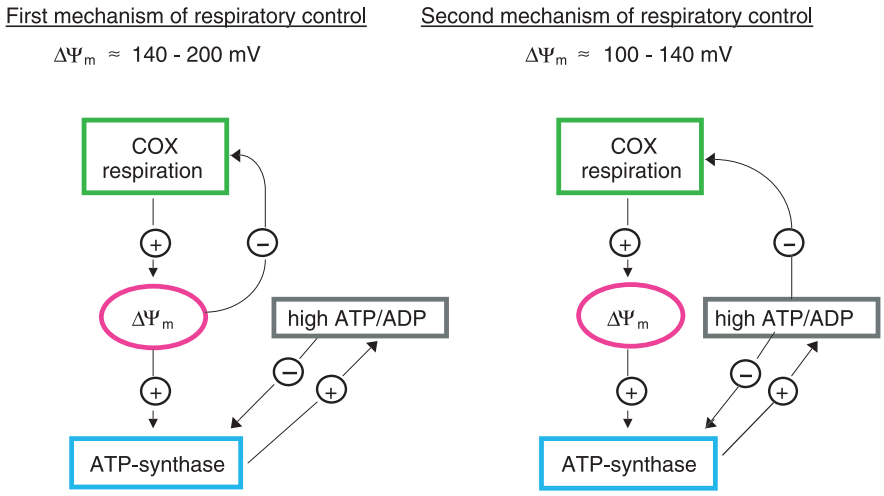

Fig. 3. Two mechanisms of respiratory control in mitochondria from vertebrates. Mitochondrial respiration is controlled by various parameters, including the reductive power at the respiratory chain (NADH/NAD^+ ratio). Here is presented the feedback control of respiration (proton pumps) by the mitochondrial membrane potential $\Delta\Psi_m$ (first mechanism of respiratory control), and the feedback control of cytochrome *c* oxidase activity by the mitochondrial ATP/ADP ratio (second mechanism of respiratory control). It is postulated that the first mechanism maintains high $\Delta\Psi_m$ values (150–200 mV), whereas the second mechanism keeps $\Delta\Psi_m$ at low levels (100–150 mV).

and at high cardiac work, 101 mV with glucose and 116 mV with pyruvate were determined. The different values between glucose and pyruvate as substrates in the perfusion medium indicate the influence of the reductive power at the respiratory chain (NADH/NAD^+ ratio) on $\Delta\Psi_m$. The decrease of $\Delta\Psi_m$ with increasing work load demonstrates its dependence on the activity of Δp consumers. The low $\Delta\Psi_m$ values in the perfused heart were corroborated in cultivated cells by Zhang et al. [94], who applied a new method using the combination of conventional fluorescence microscopy and three-dimensional deconvolution by exhaustive photon reassignment. With this method, $\Delta\Psi_m$ values of 105 ± 0.9 mV in fibroblasts and 81 ± 0.7 mV in neuroblastoma cells were measured. The low $\Delta\Psi_m$ values in cultivated cells and the working heart suggest in vivo mostly a phosphorylated cytochrome *c* oxidase, whereas the high $\Delta\Psi_m$ values in isolated mitochondria could be explained by dephosphorylation of cytochrome *c* oxidase during isolation. The question, "How could ATP be synthesized at $\Delta\Psi_m$ values below 120 mV?", will be treated in Section 7.1.1.

In vivo, high $\Delta\Psi_m$ values could occur as a consequence of stress. In fact, only after excessive muscle activity oxygen radicals have been measured by electron paramagnetic resonance (EPR) in skeletal muscle of human, rat and mouse [95]. Oxygen radicals (ROS) are produced in mitochondria only at $\Delta\Psi_m$>140 mV [96–98]. Furthermore, in several recent publications, a transitory increase of $\Delta\Psi_m$ was reported in cultured cells as a consequence of stress and after induction of apoptosis. Mitochondria hyperpolarization (increased $\Delta\Psi_m$) occurred in Caco-2 intestinal cells after induction of apoptosis by oxidized low-density lipoprotein [99]. Also sensitization of neuronal cell lines to apoptosis by overexpression of "tissue" transglutaminase leads to constitutively hyperpolarized mitochondria and increased production of ROS [100]. Finally, persistent mitochondrial hyperpolarization and increased ROS production characterize altered IL-10 signaling in patients with systemic lupus erythematosus [101].

Why does nature increase $\Delta\Psi_m$ under stress? The physiological significance of high $\Delta\Psi_m$ values may become evident from the above cited decrease of $\Delta\Psi_m$ at increasing rates of ATP utilization [93]. To maintain under high rates of ATP utilization maximal rates of ATP synthesis ($\Delta\Psi_m \approx 120$ mV), high $\Delta\Psi_m$ values (140–200 mV) would be necessary in the absence of ATP utilization. Turning off the control of respiration by the ATP/ADP ratio (second mechanism of respiratory control) would maintain high values of $\Delta\Psi_m$ and high ATP/ADP ratios.

5.3. Control of ROS production

An exponential increase of ROS production has been measured in mitochondria at membrane potentials of $\Delta\Psi_m$>140 mV [96–98]. It is generally believed that the superoxide radical anion (O_2^-) is produced by transfer of an unpaired electron from ubisemiquinone of complex III [102] or complex I [103] to molecular oxygen. The superoxide radical anion is not produced during reduction of dioxygen in cytochrome *c* oxidase, due to the almost simultaneous transfer of four electrons to molecular oxygen [104]. ROS could cause multiple diseases like Parkinson, Alzheimer [25] and cancer [105], stimulate aging [23–26] through mutations of mitochondrial DNA [106,107], and induce apoptosis by acting as signalling molecules [108,109]. It was postulated that in vivo, the second mechanism of respiratory control prevents the formation of ROS by keeping $\Delta\Psi_m$ low [65]. Under stress conditions, due to calcium-activated dephosphorylation of cytochrome *c* oxidase [80],

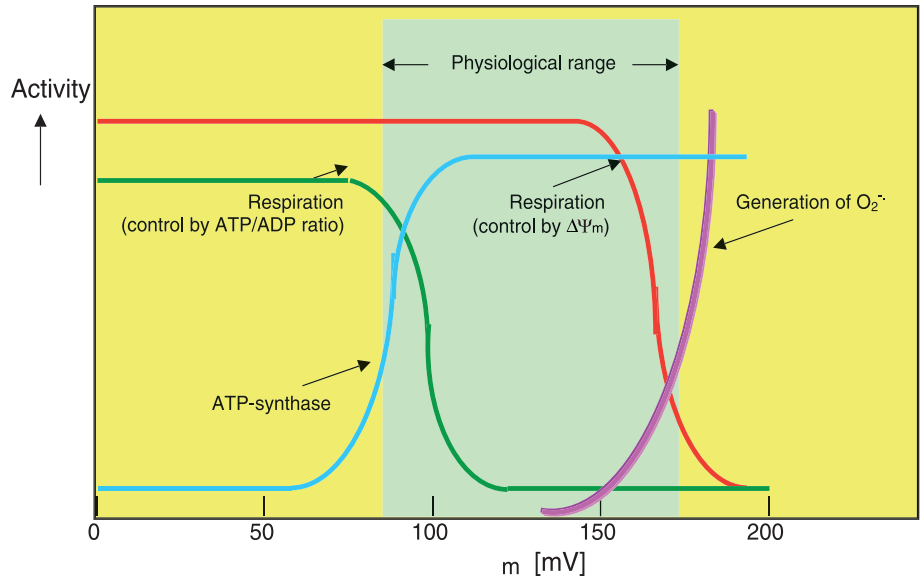

Fig. 4. Postulated control of the mitochondrial membrane potential $\Delta\Psi_m$ and ROS production. The figure presents schematically (i) the inhibition of respiration at high $\Delta\Psi_m$ via the first mechanism of respiratory control [21] (red line), (ii) the inhibition of respiration by high ATP/ADP ratios via allosteric ATP inhibition of cytochrome *c* oxidase (second mechanism of respiratory control, green line) [63], based on the membrane potential dependence of the ATP synthase [85] (blue line), and (iii) the $\Delta\Psi_m$ dependence of ROS formation [96–98] (lilac line). The suggested physiological range of $\Delta\Psi_m$ is underlaid in light green. It is proposed that in vivo "stress hormones" or agonists enhance $\Delta\Psi_m$ and ROS production via increase of cellular $[Ca^{2+}]$ and dephosphorylation of cytochrome *c* oxidase. Under relaxed conditions, $\Delta\Psi_m$ and ROS production are assumed to decrease due to hormone-stimulated increase of cAMP and phosphorylation of cytochrome *c* oxidase [65].

an increase of ROS production as a consequence of elevated $\Delta\Psi_m$ values was proposed [65,104]. In intact cells, increased ROS production has in fact been measured under various stress conditions [100,101], including the glutamate-induced neurotoxicity through elevated $[Ca^{2+}]$ [110–117].

A dramatic increase of ROS in vivo, however, could be normally prevented by the potential-dependent slip in cytochrome *c* oxidase (see Section 7.2.3), as suggested by Papa et al. [118]. In addition, recent observations of Echtay et al. [119] have shown that ROS activate the uncoupling proteins UCP1–UCP3 in the presence of fatty acids (see Section 6.3). Increased proton backflow through the uncoupling proteins would decrease $\Delta\Psi_m$ and thus prevent further production of ROS. The postulated control of $\Delta\Psi_m$ and the formation of ROS are illustrated in Fig. 4.

6. Extrinsic uncoupling of OxPhos

Extrinsic uncoupling occurs by components which increase the proton or cation permeability of membranes and thus decrease Δp in energy-conserving membranes.

6.1. Classical uncouplers

After the first description of 2,4-dinitrophenol as "uncoupler" of phosphorylation from oxidation in mitochondria by Loomis and Lipman [120], numerous other compounds were identified which uncouple OxPhos [27,121]. The most potent uncouplers are the hindered phenol SF 6847 and the hydrophobic salicylanilide S-13, which are active in vitro at concentrations in the 10-nM range [28]. The most common applied uncouplers are carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) which are active below micromolar concentrations. Classical uncouplers of OxPhos represent weak organic acids with a delocalized negative charge due to multiple mesomeric states, and are bulky hydrophobic moieties. The delocalized charge enables the molecule to penetrate biological membranes not only as protonated neutral compound but also in the charged state. The proton gradient is degraded by translocation of the negatively charged compounds out of mitochondria and back diffusion of the protonated uncouplers into the matrix.

Uncoupling could also be achieved by ionophores [122], either by combination of a uniporter with an electroneutral antiporter, or by combination of an antiporter with a uniport carrier protein of the membrane. Ionophores have been extensively applied in the characterization of membrane transport systems and in measurements of membrane potentials. Valinomycin, a carrier ionophore from *Streptomyces* specific for K^+, NH_4^+, Rb^+ and Cs^+, but not for Na^+, degrades $\Delta\Psi$ in the presence of K^+ ions. The combination of valinomycin with nigericin, an electroneutral exchanger of H^+ against K^+ (K^+>Rb^+>Na^+), which alone converts pH gradients into $\Delta\Psi$, removes completely the proton motive force. Ionomycin and A23187 are dicarboxylic ionophores with a high specificity for divalent cations. They catalyse

the electroneutral exchange of Ca^{2+} (and Mg^{2+} in the case of A23187) for two H^+ without disturbing monovalent ion gradients. In mitochondria, in the presence of Ca^{2+} ions, they act as uncouplers in combination with the native Ca^{2+} uniport transporter.

6.2. Fatty acids

Fatty acids are hydrophobic weak acids like classical uncouplers of OxPhos but act differently, because they cannot delocalize their negative charge. The uncoupling effect of long-chain fatty acids is known since almost 50 years, but the mechanism of action was not clear for long time [123–126]. Like classical uncouplers, the protonophoric action of fatty acids requires that they cross the membrane in both protonated and deprotonated forms. It has been shown that long-chain fatty acids in the undissociated form cross phosphatidylcholine bilayer with a half-time of less than 2 s, whereas this value amounts to minutes for the ionized forms [127]. Therefore, only when the outside positive membrane potential of mitochondria is high enough to drive the negatively charged fatty acids outward, they act as uncouplers of OxPhos. In liposomes, the protonophoric activity of long-chain fatty acids was measured only above a breakpoint potential of 125 mV [29].

A second mechanism of fatty acid uncoupling was suggested by Skulachev [128] via translocation of the charged anions through mitochondrial anion carriers [125]. It was proposed that the ADP/ATP-carrier and UCP1 could bind and transport out of mitochondria negatively charged fatty acids. After protonation on the cytosolic side, they could penetrate through the lipid bilayer back into the matrix as neutral fatty acids, and thus translocate protons into mitochondria. A strong argument for this mechanism of uncoupling is the fact that carboxyatractyloside, an inhibitor of the ADP/ATP-carrier, recouples in part fatty acid uncoupled mitochondria. This observation was confirmed by others [129–133], and was extended to other mitochondrial anion carriers [125,126].

We think, however, that both, the above described uncoupling by cycling of free fatty acids across the inner mitochondrial membrane at $\Delta\Psi_m$>125 mV, and the uncoupling via transport of charged fatty acids through mitochondrial anion carriers (except of the uncoupling proteins), may not occur under in vivo conditions. All measurements of uncoupling by fatty acids require concentrations of 10–100 μM of nonesterified fatty acids [134]. These high concentrations do not occur normally in vivo due to the presence of large amounts of various isoforms of fatty acid binding proteins (FABP) with dissociation constants below 1 μM [135–138]. In fact, FABP were suggested to represent one of the most abundant proteins in cells [139]. In contrast, the specific effects of very low concentrations of palmitate on activation of UCP1 (Section 6.3), and on the decrease of H^+/e^- stoichiometry in liver-type cytochrome *c* oxidase (Section 7.2.4.2), appear to be of physiological significance.

6.3. Uncoupling proteins

The uncoupling protein (UCP1, thermogenin) was identified in brown adipose tissue, based on deficient coupling of isolated mitochondria, which could be reversed by GDP (or GTP, ADP, ATP) and stimulated by fatty acids. The physiological role of UCP1 to induce thermogenesis in brown adipocytes is generally accepted [31–43]. The mechanism of uncoupling, however, remained unclear. Originally, UCP1 was suggested to solely transport protons (or OH^- ions) [33,140]. Recent studies with membrane impermeable alkylsulfonates strongly suggest, however, that uncoupling by UCP1 occurs by outward translocation of charged fatty acids through the protein and inward transport of protonated fatty acids through the lipid bilayer [141].

What remains unclear is the question on the specificity of fatty acids. In brown adipocytes from cold-adapted guinea-pig stimulation of respiration was half-maximal at 80 nM free palmitate, and half-maximal stimulation of fatty acid oxidation by mitochondria from warm-adapted and cold-adapted guinea pigs was found at 12 nM free palmitate [142]. Comparison of respiratory stimulation of brown fat mitochondria by about 10 μM of various fatty acids indicated maximal stimulation by palmitate [143]. Measurement of H^+ uptake in UCP1 proteoliposomes in the presence of 100 μM fatty acids revealed maximal effects with laurate (C12) and myristate (C14) but only half-maximal effects with decanoate (C10) and palmitate (C16) [140,144]. From these results, it appears that with increasing purity of UCP1, the specificity as well as sensitivity for stimulation of proton translocation decreases. One cofactor to increase specificity of uncoupling by UCP1 could be coenzyme Q, which was recently shown to stimulate fatty acid-dependent uncoupling by UCP1 in proteoliposomes [145]. Similar to the highly specific effect of palmitate on decreasing the H^+/e^- stoichiometry of liver-type cytochrome *c* oxidase (Section 7.2.4.2), also UCP1 might be stimulated in vivo specifically by palmitate. This conclusion is supported by results of González-Barroso et al. [146], showing stimulation of respiration of yeast mitochondria expressing UCP1 by nanomolar concentrations of palmitate, whereas micromolar concentrations were required to stimulate respiration of wild-type yeast mitochondria by fatty acids.

During the last 5 years, other uncoupling proteins were identified, based on sequence homology and mitochondrial location. UCP2 is expressed in most tissues [147,148], UCP3 mainly in heart and skeletal muscle [149,150], whereas UCP4 [151] and UCP5 (BMCP1, brain mitochondrial carrier protein 1) [152] are mainly expressed in brain. The function of UCP2–UCP5, however, is still debated (for review, see Refs. [153–156]). Recently, Echtay et al. [119] have shown that the superoxide radical anion (O_2^-) increases mitochondrial proton conductance through effects on UCP1, UCP2 and UCP3. The superoxide-induced uncoupling requires fatty acids and is inhibited by purine nucleotides.

In another report, however, these results could not be corroborated [157].

T_3 was shown to stimulate the expression of the mRNA for UCP2 in various tissues [158,159], and the UCP3 protein in heart and skeletal muscle [160]. UCP3 acts in heart and skeletal muscle as a mediator in the regulation of thermogenesis by thyroid hormones [161], and as a molecular determinant for the regulation of BMR by T_3. After a single injection of T3 to hypothyroid rats, a strict correlation between the increase of BMR, and the increase of UCP3 protein expression and fatty acid-dependent mitochondrial uncoupling was found [160]. Since T_3 also induces ROS production in mitochondria [162], the above results, obtained in vivo, would support the in vitro results of Echtay et al. [119] on activation of uncoupling proteins by the superoxide radical anion.

6.4. Proton leak of membranes

Experiments of Murphy and Brand [163,164] on the potential dependence of H^+/e^- stoichiometry of complexes IV and III of rat liver mitochondria were originally interpreted to indicate intrinsic uncoupling (slip) in cytochrome *c* oxidase but not in the cytochrome bc_1 complex. Later, the authors denied a slip and interpreted their results, and those from many other laboratories, who also demonstrated a slip in cytochrome *c* oxidase, by a natural leak of protons across the inner mitochondrial membrane [165]. Despite numerous publications by Brand et al. on the mitochondrial leak during the past 15 years, the molecular basis of the proton leak in mitochondria remained incompletely understood [30,166–169].

Rolfe and Brand [30] determined that 20–25% of the BMR in rats is due to the mitochondrial proton leak in cells and tissues. Since similar data were determined also for tissues from ectotherms, for example, in lizard hepatocytes [40], frog hepatocytes and snail hepatopancreas cells [170], thermogenesis was not assumed to represent the only function of this leak. Instead, Brand [167] suggested that the function of the proton leak in mitochondria is to decrease the production of superoxide and other ROS, by decreasing the proton motive force at the inner membrane (mainly $\Delta\Psi_m$). Mitochondrial ROS production was shown to increase exponentially with increasing $\Delta\Psi_m$ above 140 mV [96–98]. Similar conclusions were presented by Skulachev [126].

7. Intrinsic uncoupling of OxPhos ("slip")

Slip of energy conversion is principally possible in all proton pumps. The physiological significance of the regulation of slip under in vivo conditions, however, is only evident for cytochrome *c* oxidase. Whether a slip in the F_0F_1–ATP synthase is important in vivo for the regulation of ATP synthesis, or for the generation of $\Delta\Psi_m$ via hydrolysis of glycolytic ATP by the F_0F_1–ATPase, when the respiratory chain is disturbed, remains to be demonstrated.

7.1. Slip in F_0F_1–ATP synthase

F_0F_1–ATPases (or synthases) consist of a soluble F_1 domain with three catalytic sites on the $\alpha_3\beta_3$ subunit core, which is stably connected with the F_0 domain through two subunits b, forming together the "stator" ($\alpha_3\beta_3\delta ab_2$). The energy of the proton gradient, released by H^+ transport through the F_0 domain, is mechanically transferred to the catalytic $\alpha_3\beta_3$ core via the "rotor" ($\gamma\varepsilon c_n$), containing a ring of c subunits within the membrane (nomenclature of the *E. coli* enzyme) [4–6]. Thus, the F_0F_1–ATP synthase consists of two rotary motors which, depending on the demand for ATP or ion-motive force, could work in both directions. One motor operates in the forward direction, and, by rotating the central shaft, drives the other motor backwards to operate as a generator. A slip does not occur at the chemical–mechanical energy conversion in F_1 [171], but is possible at the mechanical–electrochemical energy conversion in F_0. The number of c subunits varies [172] between 10 in yeast [5] and *E. coli* [173], 11 in *Ilyobacter tartaricus* [174] and 14 in chloroplast ATPase [175]. One turn of rotation of the $\gamma\varepsilon c_n$ part yields three ATP, driven by the translocation of either protons, or Na^+ ions in the enzymes from *P. modestum* or *I. tartaricus* [84]. The H^+/ATP stoichiometry of the F_0F_1–ATP synthase, is thus variable and depends on the number of c subunits in the rotating ring. For the yeast and *E. coli* enzyme, an uneven H^+/ATP stoichiometry of 3.3 is calculated, indicating a slip (or elasticity) in the coupling between proton translocation and ATP synthesis. For the chloroplast enzyme, a higher H^+/ATP stoichiometry of 4.7 is calculated, which could explain the lower $\Delta\Psi$ required for maximal rates of the chloroplast ATP synthase, as compared to the *E. coli* enzyme [85] (see Section 5.1).

The thermodynamic stoichiometry $H^+/ATP = \Delta G_p/\Delta p$ can be calculated from the phosphorylation potential ($\Delta G_p = \Delta G^o + 2.3RT\log[ATP]/[ADP][Pi]$) and the proton motive force under the premise that ΔG_p and Δp are in thermodynamic equilibrium and delocalized into bulk phases. Recent measurements on the H^+/ATP of an aerobic thermoalkaliphilic bacillus, containing a proton-driven F_0F_1–ATP synthase, revealed variable values increasing from 2.0 at pH 7.5 to 5.7 at pH 10.0 [176]. The authors concluded that 'the growth of the bacillus correlated well with the ΔpNa^+, phosphorylation potential, and the ATP/ADP ratio, but not with Δp', indicating the predominant role of $\Delta\Psi$, and not of ΔpH, for the synthesis of ATP. It remains to be demonstrated, however, if the determined H^+/ATP ratios are correct, because the $H^+/ATP = 2.0$ at pH 7.5 would imply that the F_0F_1–ATP synthase of the bacillus contains only six c subunits in F_0 to obtain 120° rotation with two protons to yield one ATP.

7.1.1. The minimal $\Delta\Psi_m$ required to synthesize ATP in mitochondria

Can ATP be synthesized in mitochondria of living mammalian cells at $\Delta\Psi_m < 120$ mV? To answer this question, we must know the H^+/ATP stoichiometry of the mitochondrial F_0F_1–ATP synthase and the number of c subunits in F_0. The mammalian ATP synthase has probably 9–10 c subunits, corresponding to the 10 c subunits of the yeast enzyme [5]. Calculations of the thermodynamic stoichiometry H^+/ATP [22] from measurements with isolated mitochondria are questionable due to an unspecific proton leak of the inner membrane, preventing a thermodynamic equilibrium. In addition, the mitochondrial proton leak in living cells is unknown and could be different. Calculations of the phosphorylation potential ΔG_p with isolated mitochondria may not correspond to the in vivo conditions. In mitochondria from muscle and neuronal cells, the extruded ATP in the mitochondrial intermembrane space is immediately converted to ADP by creatine kinase. Therefore, nucleotides could have in vivo different "activities" in localized environments, compared to the concentrations in bulk phases of in vitro measurements. Nevertheless, if the $\Delta\Psi_m$ values of 80–120 mV, which have been measured in cultured cells [94] and perfused hearts [93], are correct, the synthesis of ATP in vivo should be possible at low mitochondrial membrane potentials. The rate of ATP synthesis by the ATP synthase of *E. coli* and *P. modestum* approaches saturation at $\Delta\Psi = 120$ mV [84,85]. The midpoint potential was approximately 70 mV for these ATP synthases, indicating their ability to synthesize ATP at rather low membrane potentials. The efficiency of ATP synthesis under suboptimal membrane potentials, however, is not known.

7.2. Slip in cytochrome c oxidase

The H^+/e^- stoichiometry in cytochrome *c* oxidase was for long time a matter of discussion [177,178]. More recently, it was generally accepted that cytochrome *c* oxidase of prokaryotes as well as of eukaryotes pumps protons at a H^+/e^- stoichiometry of 1.0 [179,180]. In addition to proton pumping, the enzyme takes up "chemical" protons from the mitochondrial matrix (or bacterial cytosol) for the reduction of dioxygen to water, which also generate $\Delta\Psi_m$ (Fig. 2). The mechanism of coupling electron transport with proton translocation in cytochrome *c* oxidase is still obscure, despite numerous investigations. Any mechanism of the proton pump should imply that translocation of an electron onto a metal center must be accompanied by movement of a proton (or cation) close to that site, to maintain electroneutrality [181].

A slip of mitochondrial proton pumps (decreased H^+/e^- stoichiometry) was first suggested by Pietrobon et al. [44,45]. The decrease of H^+/e^- stoichiometry at high proton motive force was later verified for cytochrome *c* oxidase by Azzone et al. [39], Murphy and Brand [163,164], Babcock and Wikström [179], Ferguson-Miller and Babcock [180], Papa et al. [182] and Capitanio et al. [183,184]. So far, a slip as a regulatory parameter has only been reported for complex IV, not for complex I or complex III. The recently described loss of proton pumping in the three-subunit complex III of *P. denitrificans* after binding of *N*,*N*′-dicyclohexylcarbodiimide (DCCD) or *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) to the iron sulfur protein [185] appears of no physiological relevance. The molecular basis for regulation of the slip in eukaryotic cytochrome *c* oxidase, as opposed to the bacterial enzyme, could be a different structural organization of the exit proton pathway in the mammalian enzyme, as suggested by Papa et al. [186] and Yoshikawa et al. [187]. Yoshikawa et al. assume coupling of proton pumping with the redox transition of heme a, based on variable positions of aspartate[51] in the crystal structure of the oxidized and reduced bovine heart enzyme. Aspartate[51] is assumed to represent the exit amino acid of a proton channel, because a pathway of hydrophilic amino acids and of fixed water molecules was found between heme a and aspartate[51] [18]. In contrast, from spectroscopic and kinetic studies of the bacterial enzyme, it is generally assumed that coupling between electron transport and proton pumping occurs at the binuclear heme a_3/Cu_B center, where the oxygen chemistry takes place [188–191].

Interestingly, the decrease of H^+/e^- stoichiometry in cytochrome *c* oxidase from 1.0 to 0.0 would decrease the coupling degree *q* in the respiratory chain to 0.8 (80% efficiency), since the total number of pumped protons per two electrons in the respiratory chain would decrease from 10 to 8 [22]. This corresponds nicely to the *q* value of 0.786, calculated by Stucki [42] for maximal rate of ATP synthesis. The decrease of H^+/e^- stoichiometry in cytochrome *c* oxidase to 0.5 (see Section 7.2.4) would result in a *q* value of 0.90, corresponding to the calculated *q* value of 0.91 for maximal ATP synthesis at optimal efficiency.

7.2.1. Slip by chemical modification

Chemical modification of cytochrome *c* oxidase subunits I, II and III with reagents specific for carboxyl groups in hydrophobic environs like DCCD (review in Refs. [192,193]) or EEDQ [91] resulted in a decrease of H^+/e^- stoichiometry. With EEDQ, subunits II and III and to a smaller extent subunit I of the bovine heart enzyme were labelled, but even at high reagent concentrations, the H^+/e^- ratio could not be decreased below a value of 0.25.

Also removal of subunit III of mitochondrial cytochrome *c* oxidase by detergents and high ionic strength results in decrease of H^+/e^- stoichiometry (review in Refs. [192,193]).

7.2.2. Slip by site-directed mutagenesis

Various site-directed mutations of amino acids in subunit I, which are assumed to participate in the postulated proton pathways, have been performed in cytochrome *c* oxidases of different bacteria [191,194–197]. Several mutants showed

decrease or loss of proton pumping activity of the reconstituted enzymes, suggesting the involvement of these amino acids in proton translocation. In most cases, however, also the electron transport was strongly reduced. Remarkably, the mutation of N131D in subunit I of the enzyme from *P. denitrificans* showed complete loss of proton pumping at unchanged electron transport activity [198]. The relocation in the *Rhodobacter sphaeroides* enzyme of a glutamate residue, which is assumed to play a central role in proton translocation, to the opposite side of the D-pathway [EA (I-286/IE (I-112) double mutant], resulted in 50% decrease of proton pumping stoichiometry [199]. The replacement of a carboxyl by a hydrophobic group (D132A) in subunit I of *R. sphaeroides* cytochrome *c* oxidase at the entry of the proposed proton channel, resulted in decreased electron transfer and loss of proton pumping. Although the reconstituted mutant enzyme could generate a membrane potential, which was abolished by valinomycin and CCCP, the ionophores did not stimulate, but instead inhibited respiration. The anomalous behaviour could be fully normalized by addition of fatty acids (e.g. arachidonate) [200]. It was suggested that in the mutant enzyme, protons are taken up from the positive side (intrinsic uncoupling), which is stimulated by the presence of a membrane potential. These results demonstrate, that the H^+/e^- stoichiometry of cytochrome *c* oxidase is not defined mechanistically, but rather by subtle structural restraints, and therefore could be principally variable.

7.2.3. Slip by high proton motive force

Murphy and Brand [164] investigated in rat liver mitochondria the number of translocated charges per transported electron ($q^+/2e^-$ stoichiometry) over a large range of membrane potentials $\Delta\Psi_m$ (120–180 mV), independent for cytochrome *c* oxidase and cytochrome *c* reductase (cytochrome bc_1 complex). They found no changed stoichiometry for cytochrome *c* reductase, but a decrease of H^+/e^- stoichiometry with increasing $\Delta\Psi_m$ for cytochrome *c* oxidase. The same result was obtained by Papa et al., who measured the H^+/e^- stoichiometry in the presence of the K^+-ionophore valinomycin, which converts $\Delta\Psi_m$ into ΔpH_m. They found with isolated mitochondria [182] as well as with the reconstituted enzyme [183,184] a decrease of H^+/e^- stoichiometry with increasing flow rate or increasing pH gradient with cytochrome *c* oxidase, but not with cytochrome *c* reductase. The physiological significance of a slip in cytochrome *c* oxidase at high membrane potentials was suggested to prevent the production of ROS at high $\Delta\Psi_m$ [118].

A slip of proton pumping at high membrane potentials was also concluded for bacterial cytochrome *c* oxidase by Mills and Ferguson-Miller from the effects of zinc. The activity and the efficiency of proton pumping of the reconstituted enzyme from *R. sphaeroides* are decreased by micromolar concentrations of zinc in the presence of a membrane potential. Under uncoupled conditions, millimolar concentrations of zinc are required to decrease the activity [201]. It is suggested that zinc inhibits proton movement through a proton exit path, which can allow proton back-leak (slip) at high membrane potentials [201].

7.2.4. Tissue-specific slip

Mammalian cytochrome *c* oxidase occurs in tissue-specific isozymes, differing mainly in subunits VIa, VIIa and VIII [203]. Recently, a second isoform was also described for subunit IV [204]. In heart and skeletal muscle, the heart-type isoform of subunit VIa (VIaH), in all non-skeletal muscle cells, the liver-type isoform of subunit VIa (VIaL) is expressed. Interestingly, in turkey, the liver-type of subunit VIa (VIaL) is expressed in most tissues, including heart and skeletal muscle [205]. A third isoform of subunit VIa (VIa-F) was identified in the cold-blooded fish [206].

7.2.4.1. Heart-type isozyme (subunit VIaH). With the reconstituted enzyme from bovine heart, a decrease of H^+/e^- stoichiometry from 1.0 to 0.5 was measured at increasing intraliposomal ATP/ADP ratios [53]. The decrease was half-maximal at ATP/ADP = 100, when the total intraliposomal nucleotide concentration was 5 mM and constant. The decrease of H^+/e^- stoichiometry is due to an exchange of bound ADP by ATP at the matrix domain of subunit VIaH, because it was prevented by preincubation of the enzyme with a monoclonal antibody against subunit VIaH [53,207]. The nucleotide binding site at the N-terminal domain of subunit VIaH was verified in the crystal structure of the bovine heart enzyme by identification of a cholate molecule, which is structurally very similar to ADP, and forms a salt bridge to Arg^{14} of subunit VIaH [17]. It was proposed that the physiological function of decreased H^+/e^- ratio in the heart and skeletal muscle enzyme at high ATP/ADP ratios is to stimulate thermogenesis at rest (or during sleep), when no heat is produced by muscle work and the intramitochondrial ATP/ADP ratio is expected to rise [208].

7.2.4.2. Liver-type isozyme (subunit VIaL). Reconstituted cytochrome *c* oxidase from bovine liver and kidney [206], and from turkey heart and liver [205], all containing subunit VIaL, revealed H^+/e^- stoichiometries of about 0.5 under conditions where the bovine heart enzyme exhibited a ratio of 1.0. When, however, 5% cardiolipin was added to the liposomal lipids during reconstitution, the H^+/e^- ratio of the bovine kidney enzyme increased to about 1.0. The H^+/e^- ratio of the bovine heart enzyme was not influenced by cardiolipin [56]. Preincubation of the cardiolipin-supplemented kidney proteoliposomes with palmitate decreased the H^+/e^- ratio from 1.0 to 0.5 (half-maximal effect at 0.5 μM). No other studied fatty acid or palmitoylester influenced the H^+/e^- stoichiometry of the kidney enzyme [56]. The effect of palmitate on the H^+/e^- ratio of the kidney enzyme was independent of intraliposomal nucleotides, and palmitate had no effect on the H^+/e^- ratio of the reconstituted bovine heart enzyme, suggesting that palmitate inter-

acts with the liver-type subunit VIaL. It was proposed that the physiological function of the decreased H^+/e^- ratio in non-skeletal muscle cytochrome *c* oxidase by palmitate is to stimulate thermogenesis, similar to thermogenesis in brown adipose tissue of rodents, via the noradrenalin–cAMP–fatty acid cascade, causing cold-induced thermogenesis through the uncoupling protein (UCP1) [31,34].

8. Regulatory properties of membrane protein complexes

It should be noted that the regulatory properties of isolated components of the mitochondrial membrane may not be measured with the proteins or protein complexes as isolated. Only recently, coenzyme Q was identified as an essential cofactor of the uncoupling proteins [145,209]. Regulatory properties of cytochrome *c* oxidase, like the allosteric ATP inhibition, the decrease of H^+/e^- stoichiometry of the heart-type isozyme by ATP (Section 7.2.4.1), and of the liver-type isozyme by palmitate (Section 7.2.4.2), are usually not measured with the enzyme as isolated under standard conditions for several reasons: (1) Tightly bound cholate must be exchanged by ATP or ADP via dialysis [58,59]. (2) Cardiolipin has to be added as a cofactor [54,56]. (3) Free fatty acids must be absent (e.g. half-maximal relief of allosteric ATP inhibition was measured at 0.5 μM palmitate [S. Hammerschmidt and B. Kadenbach, unpublished data]). (4) Specific regulatory properties may only be seen with the reversibly phosphorylated complexes (e.g. NADH dehydrogenase is only active in the phosphorylated state, and the allosteric ATP inhibition of cytochrome *c* oxidase is only measured with the phosphorylated enzyme). To prevent dephosphorylation of cytochrome *c* oxidase during isolation, the presence of 10 mM KF and 1 mM EGTA to the isolation media of mitochondria and the enzyme are recommended.

9. Concluding remarks

Most cellular ATP is synthesized in mitochondria by OxPhos. Both the rate and the efficiency of OxPhos are complex regulated by various parameters, including the reductive power at the respiratory chain ($NADH/NAD^+$ ratio) and the membrane potential $\Delta\Psi_m$, by interaction of allosteric effectors like ATP or ADP (i.e. the ATP/ADP ratio), free palmitate or 3,5-diiodothyronine with respiratory chain complexes, as well as by reversible phosphorylation of the complexes via hormone-induced signal cascades. The present knowledge suggests that cytochrome *c* oxidase, the terminal enzyme of the respiratory chain, is particularly involved in regulation of the rate and efficiency of OxPhos through intrinsic uncoupling. The membrane potential appears of particular importance for regulation of OxPhos. It is suggested that under relaxed physiological conditions, the membrane potential $\Delta\Psi_m$ is kept low through the potential dependence of the ATP synthase, and the allosteric ATP inhibition of phosphorylated cytochrome *c* oxidase at high ATP/ADP ratios. Under stress, when the ATP demand is high, the membrane potential is increased through dephosphorylation of cytochrome *c* oxidase and control of respiration at high $\Delta\Psi_m$ values. High mitochondrial membrane potentials, however, are accompanied by the formation of ROS (O_2^-), which could cause multiple diseases. Intrinsic uncoupling of cytochrome *c* oxidase (decrease of H^+/e^- stoichiometry from 1.0 to 0.5) is suggested to stimulate thermogenesis of endotherms in a tissue-specific manner; in heart and skeletal muscle by high intramitochondrial ATP/ADP ratios, and in non-skeletal muscle tissues by free palmitate.

The molecular basis of regulation of OxPhos in mammals remains a major challenge in bioenergetic research, because the many "supernumerary" subunits of the proton pumps, which do not occur in prokaryotes, suggest multiple interactions with allosteric effectors, accompanied by complex modulations of activity through conformational modifications. To unravel these subtle regulatory properties of the components of OxPhos, combined studies with the isolated enzyme complexes and with cultivated intact cells or tissues are required.

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References

[1] J.P. Abrahams, A.G.W. Leslie, R. Lutter, J.E. Walker, Structure at 2.8 Å resolution of F–ATPase from bovine heart mitochondria, Nature 370 (1994) 621–628.
[2] M.A. Bianchet, J. Hullihen, P.L. Pedersen, L.M. Amzel, The 2.8-Å structure of rat liver F_1–ATPase: configuration of a critical intermediate in ATP synthesis/hydrolysis, Proc. Natl. Acad. Sci. U. S. A. 95 (1994) 11065–11070.
[3] R.H. Fillingame, Coupling H^+ transport and ATP synthesis in F_1F_0–ATP synthases: glimpses of interacting parts in a dynamic molecular machine, J. Exp. Biol. 200 (1997) 217–224.
[4] W. Junge, H. Lill, S. Engelbrecht, ATP synthase: an electrochemical transducer with rotatory mechanics, Trends Biochem. Sci. 22 (1997) 420–423.
[5] D. Stock, A.G.W. Leslie, J.E. Walker, Molecular architecture of the rotary motor in ATP synthase, Science 286 (1999) 1700–1705.
[6] P. Dimroth, Operation of the F_0 motor of the ATP synthase, Biochim. Biophys. Acta 1458 (2000) 374–386.
[7] C.R. Lancaster, A. Kröger, M. Auer, H. Michel, Structure of fumarate reductase from *Wolinella siccinogenes* at 2.2 Å resolution, Nature 402 (1999) 377–385.
[8] T.M. Iverson, C. Luna-Chavez, G. Cecchini, D.C. Rees, Structure of the *Escherichia coli* fumarate reductase respiratory complex, Science 284 (1999) 1941–1942.

[9] C. Hunte, J. Koepke, C. Lange, T. Rossmanith, H. Michel, Structure at 2.3 Å resolution of the cytochrome *bc*(1) complex from the yeast *Saccharose cerevisiae* co-crystallized with an antibody Fv fragment, Structure Fold. Des. 8 (2000) 669–684.
[10] C. Lange, C. Hunte, Crystal structure of the yeast cytochrome *bc*1 complex with its bound substrate cytochrome *c*, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 2800–2805.
[11] C.-A. Yu, J.-Z. Xia, A.M. Kachurin, L. Yu, D. Xia, H. Kim, J. Deisenhofer, Crystallization and preliminary structure of beef heart mitochondrial cytochrome-*bc*1 complex, Biochim. Biophys. Acta 1275 (1996) 47–53.
[12] D. Xia, C.-A. Yu, H. Kim, J.-Z. Xia, A.M. Kachurin, L. Zhang, L. Yu, J. Deisenhofer, Crystal structure of the cytochrome *bc*1 complex from bovine heart mitochondria, Science 277 (1997) 60–66.
[13] S. Iwata, J.W. Lee, K. Okada, J.K. Lee, M. Iwata, B. Rasmussen, T.A. Link, S. Ramaswamy, B.K. Jap, Complete structure of the 11-subunit bovine mitochondrial cytochrome *bc*1 complex, Science 281 (1998) 64–71.
[14] S. Iwata, C. Ostermeier, B. Ludwig, H. Michel, Structure at 2.8 Å resolution of cytochrome *c* oxidase from *Paracoccus denitrificans*, Nature 376 (1995) 660–669.
[15] C. Ostermeier, A. Harrenga, U. Ermler, H. Michel, Structure at 2.7 Å resolution of the *Paracoccus denitrificans* two-subunit cytochrome *c* oxidase complexed with an antibody FV fragment, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 10547–10553.
[16] T. Tsukihara, H. Aoyama, E. Yamashita, T. Tomizaki, H. Shinzawa-Itoh, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, S. Yoshikawa, Structures of metal sites of oxidized bovine heart cytochrome *c* oxidase at 2.8 Å, Science 269 (1995) 1069–1074.
[17] T. Tsukihara, H. Aoyama, E. Yamashita, T. Tomizaki, H. Yamaguchi, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, S. Yoshikawa, The whole structure of the 13-subunit oxidized cytochrome *c* oxidase at 2.8 Å, Science 272 (1996) 1136–1144.
[18] S. Yoshikawa, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, E. Yamashita, N. Inoue, M. Yao, M. Jie Fei, C. Peters Libeu, T. Mizushima, H. Yamaguchi, T. Tomizaki, T. Tsukihara, Redox-coupled crystal structural changes in bovine heart cytochrome *c* oxidase, Science 280 (1998) 1723–1729.
[19] P. Mitchell, Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism, Nature 191 (1961) 144–148.
[20] P. Mitchell, Chemiosmotic coupling in oxidative and photosynthetic phosphorylation, Biol. Rev. 41 (1966) 445–502.
[21] D.G. Nicholls, S.J. Ferguson, in: Bioenergetics, 2nd edn., Academic Press Limited, London, 1992, pp. 82–87.
[22] P.C. Hinkle, M.A. Kumar, A. Resetar, D.L. Harris, Mechanistic stoichiometry of mitochondrial oxidative phosphorylation, Biochemistry 30 (1991) 3576–3582.
[23] D.C. Wallace, Diseases of the mitochondrial DNA, Annu. Rev. Biochem. 61 (1992) 1175–1212.
[24] A.W. Linnane, T. Marzuki, T. Ozawa, M. Tanaka, Mitochondrial DNA mutations as an important contributor to ageing and degenerative diseases, Lancet 1 (1989) 642–645.
[25] D.C. Wallace, Mitochondrial genetics: a paradigm for aging and degenerative diseases? Science 256 (1992) 628–632.
[26] B. Kadenbach, C. Münscher, V. Frank, J. Müller-Höcker, J. Napiwotzki, Human aging is associated with stochastic somatic mutations of mitochondrial DNA, Mutat. Res. 338 (1995) 161–172.
[27] P.G. Heytler, Uncouplers of oxidative phosphorylation, Pharmacol. Ther. 10 (1980) 461–472.
[28] H. Terada, Uncouplers of oxidative phosphorylation, Environ. Health Perspect. 87 (1990) 213–218.
[29] D. Köhnke, B. Ludwig, B. Kadenbach, A threshold membrane potential accounts for controversial effects of fatty acids on mitochondrial oxidative phosphorylation, FEBS Lett. 336 (1993) 90–94.
[30] D.F.S. Rolfe, M.D. Brand, The physiological significance of mitochondrial proton leak in animal cells and tissues, Biosci. Rep. 17 (1997) 9–16.
[31] D.G. Nicholls, R.M. Locke, Thermogenic mechanisms in brown fat, Physiol. Rev. 64 (1984) 1–64.
[32] B. Cannon, J. Nedergaard, The biochemistry of an inefficient tissue: brown adipose tissue, Essays Biochem. 20 (1985) 110–164.
[33] M. Klingenberg, Mechanism and evolution of the uncoupling protein of brown adipose tissue, Trends Biochem. Sci. 15 (1990) 108–112.
[34] J. Nedergaard, B. Cannon, The uncoupling protein thermogenin and mitochondrial thermogenesis, in: L. Ernster (Ed.), New Comprehensive Biochemistry, Molecular Mechanisms in Bioenergetics, vol. 23, Elsevier, Amsterdam, 1992, pp. 385–420.
[35] P. Bernardi, Mitochondrial transport of cations: channels, exchangers, and permeability transition, Physiol. Rev. 79 (1999) 1127–1155.
[36] S.G. Huang, S. Odoy, M. Klingenberg, Chimers of two fused ADP/ATP carrier monomers indicate a single channel for ADP/ATP transport, Arch. Biochem. Biophys. 394 (2001) 67–75.
[37] G. Fiermonte, V. Dolce, F. Palmieri, Expression in *Escherichia coli*, functional characterization, and tissue distribution of isoforms A and B of the phosphate carrier from bovine mitochondria, J. Biol. Chem. 273 (1998) 22782–22787.
[38] L. Palmieri, B. Pardo, F.M. Lasorsa, A. del Arco, K. Kobayashi, M. Iijima, M.J. Runswick, J.E. Walker, T. Saheki, J. Satrústegui, F. Palmieri, Citrin and aralar 1 are Ca^{2+}-stimulated aspartate/glutamate transporters in mitochondria, EMBO J. 20 (2001) 5060–5069.
[39] G.F. Azzone, M. Zoratti, V. Petronilli, D. Pietrobon, The stoichiometry of H^+ pumping in cytochrome *c* oxidase and the mechanism of uncoupling, J. Inorg. Biochem. 23 (1985) 349–356.
[40] M.D. Brand, P. Couture, P.L. Else, K.W. Withers, A.J. Hulbert, Evolution of energy metabolism. Proton permeability of the inner membrane of liver mitochondria is greater in a mammal than in a reptile, Biochem. J. 275 (1991) 81–86.
[41] O. Kedem, S.R. Kaplan, Degree of coupling and its relation to efficiency of energy conversion, Trans. Faraday Soc. 61 (1965) 1897–1911.
[42] J.W. Stucki, The optimal efficiency and the economic degrees of coupling of oxidative phosphorylation, Eur. J. Biochem. 109 (1980) 269–283.
[43] D.G. Nicholls, The influence of respiration and ATP hydrolysis on the proton-electrochemical gradient across the inner membrane of rat-liver mitochondria as determined by ion distribution, Eur. J. Biochem. 50 (1974) 305–315.
[44] D. Pietrobon, G.F. Azzone, D. Walz, Effect of funiculosin and antimycin A on the redox-driven H^+-pumps in mitochondria: on the nature of ''leaks'', Eur. J. Biochem. 117 (1981) 389–394.
[45] D. Pietrobon, M. Zoratti, G.F. Azzone, Molecular slipping in redox and ATPase H^+ pumps, Biochim. Biophys. Acta 723 (1983) 317–326.
[46] M. Zoratti, M. Favaron, D. Pietrobon, G.F. Azzone, Intrinsic uncoupling of mitochondrial proton pumps: 1. Non-ohmic conductance cannot account for the nonlinear dependence of static head respiration on $\Delta\mu_{H+}$, Biochemistry 25 (1986) 760–767.
[47] M.J. MacDonald, L.J. Brown, Calcium activation of mitochondrial glycerol phosphate dehydrogenase restudied, Arch. Biochem. Biophys. 326 (1996) 79–84.
[48] D.W. Gong, B.D. Weintraub, M. Reitman, Rat mitochondrial glycerol-1-phosphate dehydrogenase gene: multiple promotors, high levels in brown adipose tissue, and testis-specific regulation by thyroid hormone, DNA Cell Biol. 17 (1998) 301–309.
[49] R.W. Hendler, K. Pardhasaradhi, B. Reynafarje, B. Ludwig, Comparison of energy-transducing capabilities of the two- and three-subunit cytochromes aa_3 from *Paracoccus denitrificans* and the 13-subunit beef heart enzyme, Biophys. J. 60 (1991) 415–423.
[50] K. Pardhasaradhi, B. Ludwig, R.W. Hendler, Potentiometric and spectral studies with the two-subunit cytochrome aa_3 from *Paracoccus denitrificans*. Comparison with the 13-subunit beef heart enzyme, Biophys. J. 60 (1991) 408–414.

[51] B. Kadenbach, P. Merle, On the function of multiple subunits of cytochrome *c* oxidase from higher eucaryotes, FEBS Lett. 135 (1981) 1–11.
[52] B. Kadenbach, M. Ungibauer, J. Jarausch, U. Büge, L. Kuhn-Nentwig, The complexity of respiratory complexes, Trends Biochem. Sci. 8 (1983) 398–400.
[53] V. Frank, B. Kadenbach, Regulation of the H^+/e^- stoichiometry of cytochrome *c* oxidase from bovine heart by intraliposomal ATP/ADP ratios, FEBS Lett. 382 (1996) 121–124.
[54] S. Arnold, B. Kadenbach, Cell respiration is controlled by ATP, an allosteric inhibitor of cytochrome *c* oxidase, Eur. J. Biochem. 249 (1997) 350–354.
[55] S. Arnold, F. Goglia, B. Kadenbach, 3,5-Diiodothyronine binds to subunit Va of cytochrome *c* oxidase and abolishes the allosteric inhibition of respiration by ATP, Eur. J. Biochem. 252 (1998) 325–330.
[56] I. Lee, B. Kadenbach, Priority Paper. Palmitate decreases proton pumping of liver-type cytochrome *c* oxidase, Eur. J. Biochem. 268 (2001) 6329–6334.
[57] D.G. Nicholls, S.J. Ferguson, Bioenergetics, 3rd edn., Academic Press Limited, London, 2002, p. 121.
[58] J. Napiwotzki, K. Shinzawa-Itoh, S. Yoshikawa, B. Kadenbach, ATP and ADP bind to cytochrome *c* oxidase and regulate its activity, Biol. Chem. 378 (1997) 1013–1021.
[59] J. Napiwotzki, B. Kadenbach, Extramitochondrial ATP/ADP-ratios regulate cytochrome *c* oxidase activity via binding to the cytosolic domain of subunit IV, Biol. Chem. 379 (1998) 335–339.
[60] I. Lee, Regulation of electron transport and proton translocation in mammalian cytochrome *c* oxidase by ATP, palmitate, and protein phosphorylation. Dissertation, Fachbereich Chemie, Philipps-University Marburg, Germany, 2001, ISBN 3-89873-394-7.
[61] H.A. Lardy, H. Wellman, Oxidative phosphorylation: role of inorganic phosphate and acceptor systems in control of metabolic rates, J. Biol. Chem. 195 (1952) 215–224.
[62] B. Chance, C.M. Williams, Respiratory enzymes in oxidative phosphorylation: III. The steady state, J. Biol. Chem. 217 (1955) 405–427.
[63] B. Kadenbach, S. Arnold, Minireview. A second mechanism of respiratory control, FEBS Lett. 447 (1999) 131–134.
[64] S. Arnold, B. Kadenbach, Intramitochondrial ATP/ADP-ratios control cytochrome *c* oxidase activity allosterically, FEBS Lett. 443 (1999) 105–108.
[65] I. Lee, E. Bender, S. Arnold, B. Kadenbach, Minireview-hypothesis. New control of mitochondrial membrane potential and ROS-formation, Biol. Chem. 382 (2001) 1629–1633.
[66] K. Follmann, S. Arnold, S. Ferguson-Miller, B. Kadenbach, Cytochrome *c* oxidase from eucaryotes but not from procaryotes is allosterically inhibited by ATP, Biochem. Mol. Biol. Int. 45 (1998) 1047–1055.
[67] F. Goglia, M. Moreno, A. Lanni, Minireview. Action of thyroid hormones at the cellular level: the mitochondrial target, FEBS Lett. 452 (1999) 115–120.
[68] P.M. Yen, Physiological and molecular basis of thyroid hormone action, Physiol. Rev. 81 (2001) 1097–1142.
[69] B. Kadenbach, G. Goebell, M. Klingenberg, Differential action of thyroid hormones on enzyme levels of the DPN and TPN specific isocitrate dehydrogenase, Biochem. Biophys. Res. Commun. 14 (1963) 335–339.
[70] S. Soboll, Review. Thyroid hormone action on mitochondrial energy transfer, Biochim. Biophys. Acta 1144 (1993) 1–16.
[71] A. Lanni, M. De Felice, A. Lombardi, M. Moreno, C. Fleury, D. Ricquier, F. Goglia, Induction of UCP2 mRNA by thyroid hormones in rat heart, FEBS Lett. 418 (1997) 171–174.
[72] A. Lanni, M. Moreno, A. Lombardi, F. Goglia, 3,5-Diiodo-L-thyronine and 3,5,5′-triiodo-L-thyronine both improve the cold tolerance of hypothyroid rats, but possibly via different mechanisms, Pflügers Arch. 436 (1998) 407–414.
[73] A.C. Bianco, D. Salvatore, B. Gereben, M.J. Berry, P.R. Larsen, Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases, Endocr. Rev. 23 (2002) 38–89.
[74] R.G. Hansford, Dehydrogenase activation by Ca^{2+} in cells and tissues, J. Bioenerg. Biomembr. 23 (1991) 823–854.
[75] R.G. Hansford, D. Zorov, Role of mitochondrial calcium transport in the control of substrate oxidation, Mol. Cell. Biochem. 184 (1998) 359–369.
[76] Z. Technikova-Dobrova, A.M. Sardanelli, F. Speranza, S. Scacco, A. Signorile, V. Lorusso, S. Papa, Cyclic adenosine monophosphate-dependent phosphorylation of mammalian mitochondrial proteins: enzyme and substrate characterization and functional role, Biochemistry 40 (2001) 13941–13947.
[77] M. Thomson, Evidence of undiscovered cell regulatory mechanisms: phosphoproteins and protein kinases in mitochondria, Cell. Mol. Life Sci. 59 (2002) 213–219.
[78] S. Scacco, R. Vergari, R.C. Scarpulla, Z. Technikova-Dobrova, A. Sardanella, R. Lambo, V. Lorusso, S. Papa, cAMP-dependent phosphorylation of the nuclear encoded 18-kDa (IP) subunit of respiratory complex I and activation of the complex in serum-starved mouse fibroblast cultures, J. Biol. Chem. 275 (2000) 17578–17582.
[79] S. Papa, A.M. Sardanella, S. Scacco, V. Petruzzella, Z. Technikova-Dobrova, R. Vergari, A. Signorile, Minireview. The NADH: ubiquinone oxidoreductase (complex I) of the mammalian respiratory chain and the cAMP cascade, J. Bioenerg. Biomembr. 34 (2002) 1–10.
[80] E. Bender, B. Kadenbach, The allosteric ATP-inhibition of cytochrome *c* oxidase is reversibly switched on by cAMP-dependent phosphorylation, FEBS Lett. 466 (2000) 130–134.
[81] P.S. O'Shea, G. Petrone, R.P. Casey, A. Azzi, The current–voltage relationships of liposomes and mitochondria, Biochem. J. 219 (1984) 719–726.
[82] U. Junesch, P. Gräber, The rate of ATP synthesis as a function of $\Delta\Psi$ catalyzed by the active, reduced H+–ATPase from chloroplasts, FEBS Lett. 294 (1991) 275–278.
[83] P. Turina, B.A. Melandri, P. Gräber, ATP synthesis in chromatophores driven by artificially induced ion gradients, Eur. J. Biochem. 196 (1991) 225–229.
[84] P. Dimroth, G. Kaim, U. Matthey, Crucial role of the membrane potential for ATP synthesis by F_1F_0 ATP synthases, J. Exp. Biol. 203 (2000) 51–59.
[85] G. Kaim, P. Dimroth, ATP synthesis by F-type ATP synthase is obligatorily dependent on the transmembrane voltage, EMBO J. 18 (1999) 4118–4127.
[86] A.T. Jagendorf, E. Uribe, ATP formation caused by acid–base transition of spinach chloroplasts, Proc. Natl. Acad. Sci. U. S. A. 55 (1966) 170–177.
[87] L.D. Robb-Gaspers, P. Burnett, G.A. Rutter, R.M. Denton, R. Rizzuto, A.P. Thomas, Integrating cytosolic calcium signals into mitochondrial metabolic responses, EMBO J. 17 (1998) 4987–5000.
[88] L.D. Robb-Gaspers, G.A. Rutter, P. Burnett, G. Hajnoczky, R.M. Denton, A.P. Thomas, Coupling between cytosolic and mitochondrial calcium oscillations: role in the regulation of hepatic metabolism, Biochim. Biophys. Acta 1366 (1998) 17–32.
[89] H. Stridh, D. Gigliotti, S. Orrenius, I. Cotgreave, The role of calcium in pre- and postmitochondrial events in tributyltin-induced T-cell apoptosis, Biochem. Biophys. Res. Commun. 266 (1999) 460–465.
[90] R.P. Hafner, C.D. Nobes, A.D. McGown, M.D. Brand, Altered relationship between proton motive force and respiration rate in non-phosphorylating liver mitochondria isolated from rats of different thyroid hormone status, Eur. J. Biochem. 178 (1988) 511–518.
[91] D. Steverding, B. Kadenbach, Influence of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline modification on proton translocation and membrane potential of reconstituted cytochrome *c* oxidase support "proton slippage", J. Biol. Chem. 266 (1991) 8097–8101.

[92] D. Steverding, D. Köhnke, B. Ludwig, B. Kadenbach, Proton slippage in cytochrome *c* oxidase of *Paracoccus denitrificans*. Membrane potential measurements with the two- and three-subunit enzyme, Eur. J. Biochem. 212 (1993) 827–831.
[93] B. Wan, C. Doumen, J. Duszynski, G. Salama, T.C. Vary, K.F. LaNoue, Effects of cardiac work on electrical potential gradient across mitochondrial membrane in perfused rat hearts, Am. J. Physiol. 265 (1993) H453–H460.
[94] H. Zhang, H.M. Huang, R.C. Carson, J. Mahmood, H.M. Thomas, G.E. Gibson, Assessment of membrane potentials of mitochondrial populations in living cells, Anal. Biochem. 298 (2001) 170–180.
[95] M.J. Jackson, R.H. Edwards, M.C. Symons, Electron spin resonance studies of intact mammalian skeletal muscle, Biochim. Biophys. Acta 847 (1985) 185–190.
[96] S.S. Liu, Generating, partitioning, targeting and functioning of superoxide in mitochondria, Biosci. Rep. 17 (1997) 259–272.
[97] S.S. Liu, Cooperation of a "reactive oxygen cycle" with the Q cycle and the proton cycle in the respiratory chain—superoxide generating and cycling mechanism in mitochondria, J. Bioenerg. Biomembr. 31 (1999) 367–376.
[98] S.S. Korshunov, V.P. Skulachev, A.A. Starkov, High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria, FEBS Lett. 416 (1997) 15–18.
[99] C. Giovannini, P. Matarrese, B. Scazzocchio, M. Sanchez, R. Masella, W. Malorni, Mitochondria hyperpolarization is an early event in oxidized low-density lipoprotein-induced apoptosis in Caco-2 intestinal cells, FEBS Lett. 523 (2002) 200–206.
[100] M. Piacentini, M.G. Farrace, L. Piredda, P. Matarrese, F. Falasca, L. Falasca, C. Rodolfo, A.M. Giammarioli, E. Verderio, M. Malorni, W. Malorni, Transglutaminase overexpression sensitizes neuronal cell lines to apoptosis by increasing mitochondrial membrane potential and cellular oxidative stress, J. Neurochem. 81 (2002) 1061–1072.
[101] P. Gergely Jr., B. Niland, N. Gonchoroff, R. Pullmann Jr., P.E. Phillips, A. Perl, Persistent mitochondrial hyperpolarization, increased reactive oxygen intermediate production, and cytoplasmic alkalinization characterize altered IL-10 signaling in patients with systemic lupus erythematosus, J. Immunol. 169 (2002) 1092–1101.
[102] H.J. Forman, A. Azzi, Production of reactive oxygen species in mitochondria and age-associated pathophysiology: a reality check, in: E. Cadenas, L. Packer (Eds.), Understanding the Process of Aging, The Roles of Mitochondria, Free Radicals, and Antioxidants, Marcel Dekker, Inc., New York, 1999, pp. 73–94.
[103] G. Lenaz, C. Bovina, M. D'Aurelio, R. Fato, G. Formiggini, M.L. Genova, G. Giuliano, M.M. Pich, U. Paolucci, G.P. Castelli, B. Ventura, Role of mitochondria in oxidative stress and aging, Ann. N.Y. Acad. Sci. 959 (2002) 199–213.
[104] B. Ludwig, E. Bender, S. Arnold, M. Hüttemann, I. Lee, B. Kadenbach, Cytochrome *c* oxidase and the regulation of oxidative phosphorylation, ChemBioChem 2 (2001) 392–403.
[105] Y.-A. Suh, R.S. Arnold, B. Lassegue, J. Shi, X. Xu, D. Sorescu, A.B. Chung, K.K. Griendling, J.D. Lambeth, Cell transformation by superoxide-generating oxidase Mox1, Nature 401 (1999) 79–82.
[106] C. Richter, Reactive oxygen and DNA damage in mitochondria, Mutat. Res. 275 (1992) 249–255.
[107] B.N. Ames, M.K. Shigenaga, T.M. Hagen, Oxidants, antioxidants and the degenerative diseases of aging, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 7915–7922.
[108] H.U. Simon, A. Haj-Yehia, F. Levi-Schaffer, Role of reactive oxygen species (ROS) in apoptosis induction, Apoptosis 5 (2000) 415–418.
[109] R.J. Carmody, T.G. Cotter, Signalling apoptosis: a radical approach, Redox Rep. 6 (2001) 77–90.
[110] L.L. Dugan, S.L. Sensi, L.M. Canzoniero, S.M. Handran, T.S. Lin, M.P. Goldberg, D.W. Choi, Mitochondrial production of reactive oxygen species in corticol neurons following exposure to *N*-methyl-D-aspartate, J. Neurosci. 15 (1995) 6377–6388.
[111] I.J. Reynolds, T.G. Hastings, Glutamate induces the production of reactive oxygen species in cultured forebrain neurons following NMDA receptor activation, J. Neurosci. 15 (1995) 3318–3327.
[112] V.P. Bindokas, J. Jordan, C.C. Lee, R.J. Miller, Superoxide production in rat hippocampal neurons: selective imaging with hydroethidine, J. Neurosci. 16 (1996) 1324–1336.
[113] J.L.P. Velazquez, M.V. Frantseva, P.L. Carlen, In vitro ischemia promotes glutamate-mediated free radical generation and intracellular calcium accumulation in hippocampal pyramidal neurons, J. Neurosci. 17 (1997) 9085–9094.
[114] R.F. Castilho, O. Hansson, M.W. Ward, S.L. Budd, D.G. Nicholls, Mitochondrial control of acute glutamate excitotoxicity in cultured cerebellar granule cells, J. Neurosci. 18 (1998) 10277–10286.
[115] B. Sengpiel, E. Preis, J. Krieglstein, J.H. Prehn, NMDA-induced superoxide production and neurotoxicity in cultured rat hippocampal neurons: role of mitochondria, Eur. J. Neurosci. 10 (1998) 1903–1910.
[116] A.A. Boldyrev, D.O. Carpenter, M.J. Huentelman, C.M. Peters, P. Johnson, Sources of reactive oxygen species production in excitotoxin-stimulated cerebellar granule cells, Biochem. Biophys. Res. Commun. 256 (1999) 320–324.
[117] O. Vergun, A.I. Sobolevsky, M.V. Yelshansky, J. Keelan, B.I. Khodorov, M.R. Duchen, Exploration of the role of reactive oxygen species in glutamate neurotoxicity in rat hippocampal neurones in culture, J. Physiol. 531 (2001) 147–163.
[118] S. Papa, F. Guerrieri, N. Capitanio, A possible role of slips in cytochrome *c* oxidase in the antioxygen defense system of the cell, Biosci. Rep. 17 (1997) 23–31.
[119] K.S. Echtay, D. Roussel, J. St. Pierre, M.B. Jekabsons, S. Cadenas, J.A. Stuart, J.A. Harper, S.J. Roebuck, A. Morrison, S. Pickering, J.C. Clapham, M.D. Brand, Superoxide activates mitochondrial uncoupling proteins, Nature 415 (2002) 96–99.
[120] W.F. Loomis, F. Lipman, Reversible inhibition of the coupling between phosphorylation and oxidation, J. Biol. Chem. 173 (1948) 807–808.
[121] E.C. Weinbach, J. Garbus, Mechanism of action of reagents that uncouple oxidative phosphorylation, Nature 221 (1969) 1016–1018.
[122] B.C. Pressman, Biological applications of ionophores, Annu. Rev. Biochem. 45 (1976) 501–530.
[123] L. Wojtczak, Effect of long-chain fatty acids and acyl-CoA on mitochondrial permeability, transport, and energy-coupling processes, J. Bioenerg. Biomembr. 8 (1976) 293–311.
[124] H. Rottenberg, Decoupling of oxidative phosphorylation and photophosphorylation, Biochim. Biophys. Acta 1018 (1990) 1–17.
[125] L. Wojtczak, M.R. Wieckowski, The mechanisms of fatty acid-induced proton permeability of the inner mitochondrial membrane, J. Bioenerg. Biomembr. 31 (1999) 447–455.
[126] V.P. Skulachev, Uncoupling: new approaches to an old problem of bioenergetics, Biochim. Biophys. Acta 1363 (1998) 100–124.
[127] F. Kamp, J.A. Hamilton, pH gradients across phospholipid membranes caused by fast flip-flop on unionized fatty acids, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 11367–11370.
[128] V.P. Skulachev, Hypothesis. Fatty acid circuit as a physiological mechanism of uncoupling of oxidative phosphorylation, FEBS Lett. 294 (1991) 158–162.
[129] P. Schönfeld, Does the function of adenine nucleotide translocase in fatty acid uncoupling depend on the type of mitochondria? FEBS Lett. 264 (1990) 246–248.
[130] C. Valcarce, J.M. Cuezva, Interaction of adenine nucleotides with the adenine nucleotide translocase regulates the developmental changes in proton conductance of the inner mitochondrial membrane, FEBS Lett. 294 (1991) 225–228.
[131] A. Vianello, E. Petrussa, F. Macri, ATP/ADP antiporter is involved in uncoupling of plant mitochondria induced by low concentrations of palmitate, FEBS Lett. 347 (1994) 239–242.
[132] P. Schönfeld, P. Jezek, E.A. Belyaeva, J. Borecky, V.S. Slyshenkov, M.R. Wieckowski, V.P. Wojczak, Photomodification of mitochondrial proteins by azido fatty acids and its effect on mitochon-

drial energetics. Further evidence for the role of the ADP/ATP carrier in fatty-acid-mediated uncoupling, Eur. J. Biochem. 240 (1996) 387–393.
[133] N.N. Brustovetsky, M. Klingenberg, The reconstituted ADP/ATP carrier can mediate H^+ transport by free fatty acids, which is further stimulated by mersalyl, J. Biol. Chem. 269 (1994) 27329–27336.
[134] P. Schönfeld, L. Schild, W. Kunz, Long-chain fatty acids act as protonophoric uncouplers of oxidative phosphorylation in rat liver mitochondria, Biochim. Biophys. Acta 977 (1989) 266–272.
[135] T. Börchers, F. Spener, Fatty acid binding proteins, Curr. Top. Membr. 40 (1994) 261–294.
[136] L. Banaszak, N. Winter, Z. Xu, D.A. Bernlohr, S. Cowan, T.A. Jones, Lipid-binding proteins: a family of fatty acid and retinoid transport proteins, Adv. Protein Chem. 45 (1994) 89–151.
[137] J.H. Veerkamp, R.G. Maatman, Cytoplasmic fatty acid-binding proteins: their structure and genes, Prog. Lipid Res. 34 (1995) 17–52.
[138] F.G. Schaap, G.J. van der Vusse, J.F.C. Glatz, Fatty acid-binding proteins in the heart, Mol. Cell. Biochem. 180 (1998) 43–51.
[139] F. Spener, T. Börchers, M. Mukherjea, On the role of fatty acid binding proteins in fatty acid transport and metabolism, FEBS Lett. 244 (1989) 1–5.
[140] M. Klingenberg, S.-G. Huang, Structure and function of the uncoupling protein from brown adipose tissue, Biochim. Biophys. Acta 1415 (1999) 271–296.
[141] M. Jaburek, M. Varecha, P. Jezek, K.D. Garlid, Alkylsulfonates as probes of uncoupling protein transport mechanism. Ion pair transport demonstrates that direct H^+ translocation by UCP1 is not necessary for uncoupling, J. Biol. Chem. 276 (2001) 31897–31905.
[142] S.A. Cunningham, H. Wiesinger, D.G. Nicholls, Quantification of fatty acid activation of the uncoupling protein in brown adipocytes and mitochondria from guinea-pig, Eur. J. Biochem. 157 (1986) 415–420.
[143] E. Rial, A. Poustie, D.G. Nicholls, Brown-adipose-tissue mitochondria: the regulation of the 32 000-Mr uncoupling protein by fatty acids and purine nucleotides, Eur. J. Biochem. 137 (1983) 197–203.
[144] E. Winkler, M. Klingenberg, Effect of fatty acids on H^+ transport activity of the reconstituted uncoupling protein, J. Biol. Chem. 269 (1994) 2508–2515.
[145] K.S. Echtay, E. Winkler, M. Klingenberg, Coenzyme Q is an obligatory cofactor for uncoupling protein function, Nature 408 (2000) 609–613.
[146] M.M. González-Barroso, C. Fleury, F. Bouillaud, D.G. Nicholls, E. Rial, The uncoupling protein UCP1 does not increase the proton conductance of the inner mitochondrial membrane by functioning as a fatty acid anion transporter, J. Biol. Chem. 273 (1998) 15528–15532.
[147] C. Fleury, M. Neverova, S. Collins, S. Raimbault, O. Champigny, C. Levi-Meyrueis, F. Bouillaud, M.F. Seldin, R.S. Surwit, D. Ricquier, C.H. Warden, Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia, Nat. Genet. 15 (1997) 269–272.
[148] R.E. Gimeno, M. Dembski, X. Weng, N. Deng, A.W. Shyan, C.J. Gimeno, F. Iris, S.J. Ellis, E.A. Woolf, L.A. Tartaglia, Cloning and characterization of an uncoupling protein homolog: a potential molecular mediator of human thermogenesis, Diabetes 46 (1997) 900–906.
[149] O. Boss, C. Samec, A. Paoloni-Giacobino, C. Rossier, A. Dulloo, J. Sejdoux, P. Muzzin, J.P. Giacobino, Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression, FEBS Lett. 408 (1997) 39–42.
[150] A. Vidal-Puig, G. Solanes, D. Grujic, J.S. Flier, B.B. Lowell, UCP3: an uncoupling protein homologue expressed preferentially and abundantly in skeletal muscle and brown adipose tissue, Biochem. Biophys. Res. Commun. 235 (1997) 79–82.
[151] W. Mao, X.X. Yu, A. Zhong, W. Li, J. Brush, S.W. Sherwood, S.H. Adams, G. Pan, UCP4, a novel brain-specific mitochondrial protein that reduces membrane potential in mammalian cells, FEBS Lett. 443 (1999) 326–330.
[152] D. Sanchis, C. Fleury, N. Chomiki, M. Goebern, Q. Huang, M. Neverova, F. Grégoire, J. Easlick, S. Raimbault, C. Lévi-Meyrueis, B. Miroux, S. Collins, M. Seldin, D. Richard, C. Warden, F. Bouillaud, D. Ricquier, BMCP1, a novel mitochondrial carrier with high expression in the central nervous system of humans and rodents, and respiration uncoupling activity in recombinant yeast, J. Biol. Chem. 273 (1998) 36411–36415.
[153] C. Pecqueur, E. Couplan, F. Bouillaud, D. Ricquier, Genetic and physiological analysis of the role of uncoupling proteins in human energy homeostasis, J. Mol. Med. 79 (2001) 48–56.
[154] S. Collins, W. Cao, K.W. Daniel, T.M. Dixon, A. Medvedev, H. Onuma, R. Surwit, Adrenoceptors, uncoupling proteins, and energy expenditure, Exp. Biol. Med. 226 (2001) 982–990.
[155] S.H. Adams, G. Pan, X.X. Yu, Perspectives on the biology of uncoupling protein (UCP) homologues, Biochem. Soc. Trans. 29 (2001) 798–802.
[156] G. Argyropoulos, M.E. Harper, Uncoupling proteins and thermoregulation, J. Appl. Physiol. 92 (2002) 2187–2198.
[157] E. Couplan, M. Gonzalez-Barroso, M.C. Alves-Guerra, D. Ricquier, M. Goubern, F. Bouillaud, No evidence for a basal, retinoic or superoxide-induced uncoupling activity of the UCP2 present in spleen or lung mitochondria, J. Biol. Chem. 277 (2002) 26268–26275.
[158] A. Lanni, M. Moreno, A. Lombardi, F. Goglia, Calorigenic effect of diiodothyronines in the rat, J. Physiol. (Lond.) 494 (1996) 831–837.
[159] T. Masaki, H. Yoshimatsu, T. Kakume, S. Hidaka, M. Kurokawa, T. Sakata, Enhanced expression of uncoupling protein 2 gene in rat white adipose tissue and skeletal muscle following chronic treatment with thyroid hormone, FEBS Lett. 418 (1997) 323–326.
[160] P. De Lange, A. Lanni, L. Beneduce, M. Moreno, A. Lombardi, E. Silvestri, F. Goglia, Uncoupling protein-3 is a molecular determinant for the regulation of resting metabolic rate by thyroid hormone, Endocrinology 143 (2001) 504–510.
[161] D.W. Gong, Y. He, M. Karas, M. Reitman, Uncoupling protein-3 is a mediator of thermogenesis regulated by thyroid hormone, beta3-adrenergic agonists, and leptin, J. Biol. Chem. 272 (1997) 24129–24132.
[162] L.A. Videla, V. Fernandez, Influence of hyperthyroidism on superoxide radical and hydrogen peroxide production by rat liver submitochondrial particles, Free Radic. Res. Commun. 18 (1993) 329–335.
[163] M.P. Murphy, M.D. Brand, Variable stoichiometry of proton pumping by the mitochondrial respiratory chain, Nature 329 (1987) 170–172.
[164] M.P. Murphy, M.D. Brand, The stoichiometry of charge translocation by cytochrome *c* oxidase and the cytochrome bc_1 complex of mitochondria at high membrane potential, Eur. J. Biochem. 173 (1988) 645–651.
[165] M.D. Brand, L.-F. Chien, P. Diolet, Experimental discrimination between proton leak and redox slip during mitochondrial electron transport, Biochem. J. 297 (1994) 27–29.
[166] M.D. Brand, K.M. Brindle, J.A. Buckingham, J.A. Harper, D.F.S. Rolfe, J.A. Stuart, The significance and mechanism of mitochondrial proton conductance, Int. J. Obes. 23 (Suppl. 6) (1999) S4–S11.
[167] M.D. Brand, Uncoupling to survive? The role of mitochondrial inefficiency in ageing, Exp. Gerontol. 35 (2000) 811–820.
[168] J.A. Stuart, K.M. Brindle, J.A. Harper, M.D. Brand, Mitochondrial proton leak and the uncoupling proteins, J. Bioenerg. Biomembr. 31 (1999) 517–525.
[169] J.A. Stuart, S. Cadenas, M.B. Jekabsons, D. Roussel, M.D. Brand, Mitochondrial proton leak and the uncoupling protein 1 homologues, Biochim. Biophys. Acta 1504 (2001) 144–158.
[170] M. Guppy, D.C. Reeves, T. Bishop, P. Withers, J.A. Brand, M.D. Brand, Intrinsic metabolic depression in cells isolated from the hepatopancreas of estivating snails, FASEB J. 14 (2000) 999–1004.
[171] W. Junge, O. Pänke, D.A. Cherepanov, K. Gumbiowski, M. Müller, S. Engelbrecht, Inter-subunit rotation and elastic power transmission in F_0F_1–ATPase, FEBS Lett. 504 (2001) 152–160.

[172] S.J. Ferguson, ATP synthase: what dictates the size of a ring? Curr. Biol. 10 (2000) R804–R808.
[173] W. Jiang, J. Hermolin, R.H. Fillingame, The preferred stoichiometry of *c* subunits in the rotary motor sector of *Escherichia coli* ATP synthase is 10, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 4966–4971.
[174] H. Stahlberg, D.J. Müller, K. Suda, D. Fotiadis, A. Engel, T. Meier, U. Matthey, P. Dimroth, Bacterial Na+–ATP synthase has an undecameric rotor, EMBO Rep. 21 (2001) 229–233.
[175] H. Seelert, A. Poetsch, N.A. Dencher, A. Engel, H. Stahlberg, D. Müller, Proton-powered turbine of a plant motor, Nature 405 (2000) 418–419.
[176] K. Olsson, S. Keis, H.W. Morgan, P. Dimroth, G.M. Cook, Bioenergetic properties of the thermoalkaliphilic *Bacillus* sp. strain TA2.A1, J. Bacteriol. 185 (2003) 461–465.
[177] M. Wikström, Pumping of protons from the mitochondrial matrix by cytochrome *c* oxidase, Nature 308 (1984) 558–560.
[178] B. Reynafarje, L.E. Costa, A.L. Lehninger, Upper and lower limits of the proton stoichiometry of cytochrome *c* oxidation in rat liver mitoplasts, J. Biol. Chem. 261 (1986) 8254–8262.
[179] G.T. Babcock, M. Wikström, Oxygen activation and the conservation of energy in cell respiration, Nature 356 (1992) 301–309.
[180] S. Ferguson-Miller, G.T. Babcock, Heme/copper terminal oxidases, Chem. Rev. 96 (1996) 2889–2907.
[181] P.R. Rich, B. Meunier, R. Mitchell, A.J. Moody, Coupling of charge and proton movement in cytochrome *c* oxidase, Biochim. Biophys. Acta 1275 (1996) 91–95.
[182] S. Papa, N. Capitanio, G. Capitanio, E. De Nitto, M. Minuto, The cytochrome chain of mitochondria exhibits variable H^+/e^- stoichiometry, FEBS Lett. 288 (1991) 183–186.
[183] N. Capitanio, G. Capitanio, E. De Nitto, G. Billani, S. Papa, H^+/e^- stoichiometry of mitochondrial cytochrome complexes reconstituted in liposomes. Rate-dependent changes of the stoichiometry in the cytochrome *c* oxidase vesicles, FEBS Lett. 288 (1991) 179–182.
[184] N. Capitanio, G. Capitanio, D.A. Demarinis, E. De Nitto, S. Massari, S. Papa, Factors affecting the H^+/e^- stoichiometry in mitochondrial cytochrome *c* oxidase: influence of the rate of electron flow and transmembrane ΔpH^+, Biochemistry 35 (1996) 10800–10806.
[185] T. Cocco, G. Cutecchia, B. Ludwig, M. Korn, S. Papa, M. Lorusso, Carboxyl residues in the iron–sulfur protein are involved in the proton pumping activity of *P. denitrificans bc* (1) complex, Biochemistry 40 (2001) 15396–15402.
[186] S. Papa, N. Capitanio, G. Villani, G. Capitanio, A. Bizzoca, L.L. Palese, V. Carlino, E. De Nitto, Cooperative coupling and role of heme a in the proton pump of heme–copper oxidases, Biochimie 80 (1998) 821–836.
[187] S. Yoshikawa, K. Shinzawa-Itoh, T. Tsukihara, X-ray structure and the reaction mechanism of bovine heart cytochrome *c* oxidase, J. Inorg. Biochem. 82 (2000) 1–7.
[188] H. Michel, Cytochrome *c* oxidase: catalytic cycle and mechanisms of proton pumping—a discussion, Biochemistry 38 (1999) 15129–15140.
[189] L. Florens, C. Hoganson, J. McCracken, J. Fetter, D. Mills, G.T. Babcock, S. Ferguson-Miller, The role of magnesium and its associated water channel in activity and regulation of cytochrome *c* oxidase, in: G. Peschek, W. Loeffelhardt, G. Schmetterer (Eds.), The Phototropic Procaryotes, Kluwer Academic/Plenum Press, New York, 1999, pp. 329–339.
[190] M. Wikström, Mechanism of proton translocation by cytochrome *c* oxidase: a new four-stroke histidine cycle, Biochim. Biophys. Acta 1458 (2000) 188–198.
[191] D.A. Mills, L. Florens, C. Hiser, J. Qian, S. Ferguson-Miller, Where is 'outside' in cytochrome *c* oxidase and how and when do protons get there? Biochim. Biophys. Acta 1458 (2000) 180–187.
[192] L.J. Prochaska, P.S. Fink, On the role of subunit III in proton translocation in cytochrome *c* oxidase, J. Bioenerg. Biomembr. 19 (1987) 143–166.
[193] M.P. Murphy, Slip and leak in mitochondrial oxidative phosphorylation, Biochim. Biophys. Acta 977 (1989) 123–141.
[194] U. Pfitzner, A. Odenwald, T. Ostermann, L. Weingard, B. Ludwig, O.-M.H. Richter, Cytochrome *c* oxidase (heme aa_3) from *Paracoccus denitrificans*: analysis of mutations in putative proton channels of subunit I, J. Bioenerg. Biomembr. 30 (1998) 89–97.
[195] H.-M. Lee, T.K. Das, D.L. Rousseau, D.A. Mills, S. Ferguson-Miller, R.B. Gennis, Mutations in the putative H-channel in the cytochrome *c* oxidase from *Rhodobacter sphaeroides* show that this channel is not important for proton conduction but reveal modulation of the properties of heme a, Biochemistry 39 (2000) 2989–2996.
[196] D. Zaslavsky, R.B. Gennis, Proton pumping by cytochrome oxidase: progress, problems and postulates, Biochim. Biophys. Acta 1458 (2000) 164–179.
[197] M.M. Pereira, C.M. Gomes, M. Teixeira, Plasticity of proton pathways in haem–copper oxygen reductases, FEBS Lett. 522 (2002) 14–18.
[198] U. Pfitzner, K. Hoffmeier, A. Harrenga, A. Kannt, H. Michel, E. Bambeerg, O.-M.H. Richter, B. Ludwig, Tracing the D-channel in reconstituted site-directed mutants of cytochrome *c* oxidase from *Paracoccus denitrificans*, Biochemistry 39 (2000) 6756–6762.
[199] G. Gilderson, A. Aagaard, P. Brzezinski, Relocation of an internal proton donor in cytochrome *c* oxidase results in an altered p*K*(a) and a non-integer pumping stoichiometry, Biophys. Chem. 98 (2002) 105–114.
[200] J. Fetter, M. Sharpe, J. Qian, D. Mills, S. Ferguson-Miller, P. Nicholls, Fatty acids stimulate activity and restore respiratory control in a proton channel mutant of cytochrome *c* oxidase, FEBS Lett. 393 (1996) 155–160.
[201] D.A. Mills, B. Schmidt, C. Hiser, E. Westley, S. Ferguson-Miller, Membrane potential-controlled inhibition of cytochrome *c* oxidase by zinc, J. Biol. Chem. 277 (2002) 14894–14901.
[202] D.A. Mills, S. Ferguson-Miller, Influence of structure, pH, and membrane potential on proton movement in cytochrome *c* oxidase, Biochim. Biophys. Acta 1555 (2002) 96–100.
[203] L.I. Grossman, M.I. Lomax, Review. Nuclear genes for cytochrome *c* oxidase, Biochim. Biophys. Acta 1352 (1997) 174–192.
[204] M. Hüttemann, B. Kadenbach, L.I. Grossman, Mammalian subunit IV isoforms of cytochrome *c* oxidase, Gene 267 (2001) 111–123.
[205] M. Hüttemann, S. Arnold, I. Lee, N. Mühlenbein, D. Linder, F. Lottspeich, B. Kadenbach, Turkey cytochrome *c* oxidase contains subunit VIa of the liver-type associated with low efficiency of energy transduction, Eur. J. Biochem. 267 (2000) 2098–2104.
[206] M. Hüttemann, V. Frank, B. Kadenbach, The possible role of isoforms of cytochrome *c* oxidase subunit VIa in mammalian thermogenesis, Cell. Mol. Life Sci. 55 (1999) 1482–1490.
[207] G. Anthony, A. Reimann, B. Kadenbach, Tissue-specific regulation of bovine heart cytochrome *c* oxidase by ADP via interaction with subunit VIa, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 1652–1656.
[208] F. Rohdich, B. Kadenbach, Tissue-specific regulation of cytochrome *c* oxidase efficiency by nucleotides, Biochemistry 32 (1993) 8499–8503.
[209] K.S. Echtay, E. Winkler, K. Frischmuth, M. Klingenberg, Uncoupling proteins 2 and 3 are highly active H^+ transporters and highly nucleotide sensitive when activated by coenzyme Q (ubiquinone), Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 1416–1421.