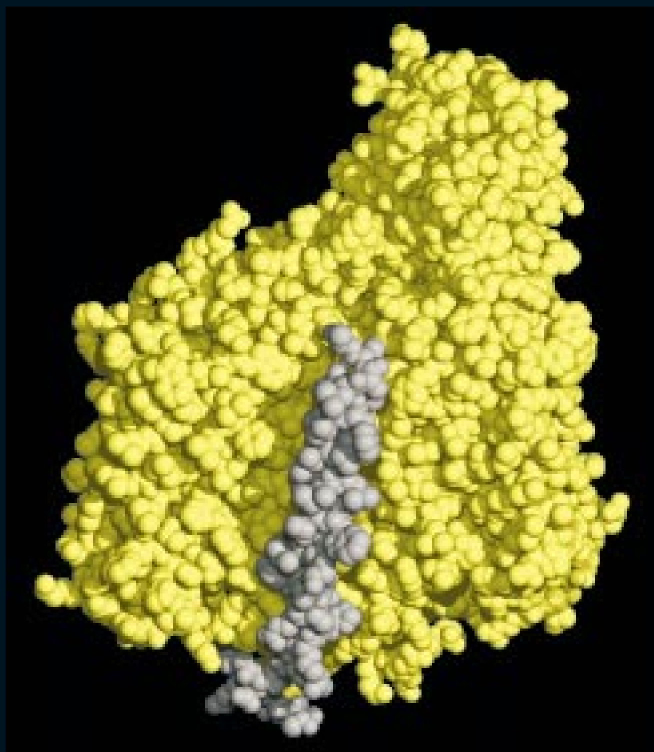
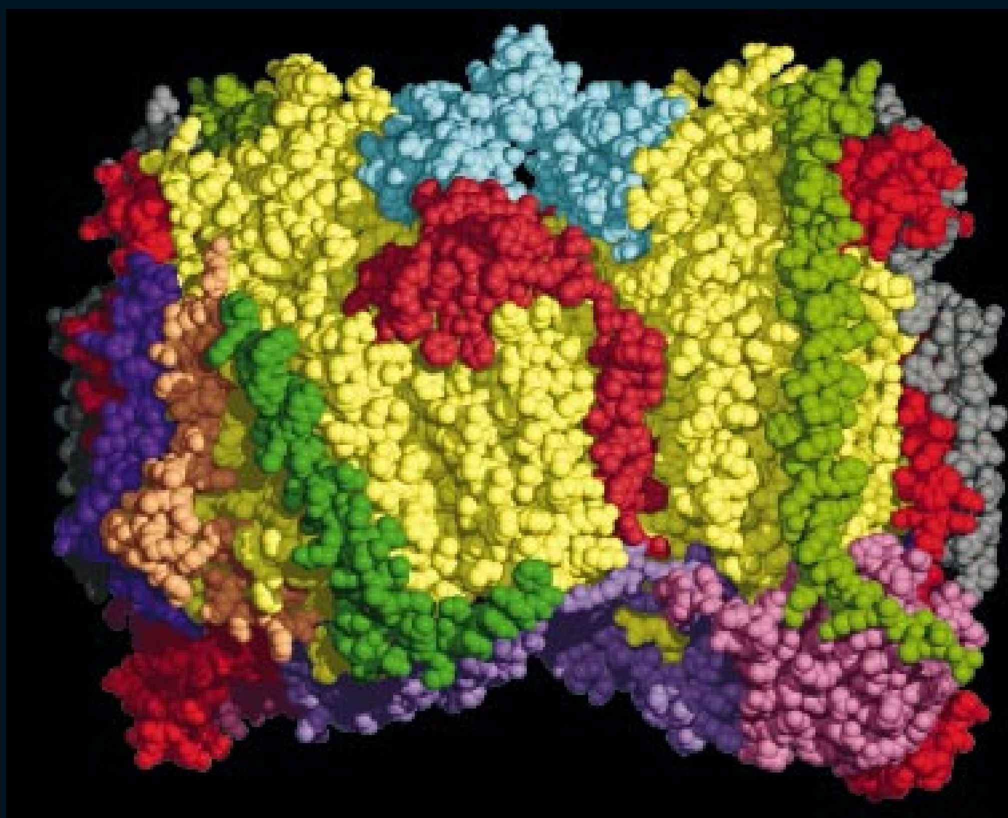
Evolution of the respiratory enzyme cytochrome *c* oxidase



The three catalytic subunits of the enzyme from *Paracoccus denitrificans* (top) and from beef heart (bottom), shown in yellow, are very similar. The dramatic change during evolution follows from the dimerization and addition of ten regulatory subunits, shown in different colors, to the mammalian enzyme. The bacterial enzyme contains one regulatory subunit (gray).



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Cytochrome *c* Oxidase and the Regulation of Oxidative Phosphorylation

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Life of higher organisms is essentially dependent on the efficient synthesis of ATP by oxidative phosphorylation in mitochondria. An important and as yet unsolved question of energy metabolism is how are the variable rates of ATP synthesis at maximal work load during exercise or mental work and at rest or during sleep regulated. This article reviews our present knowledge on the structure of bacterial and eukaryotic cytochrome c *oxidases and correlates it with recent results on the regulatory functions of nuclear-coded subunits of the eukaryotic enzyme, which are absent from the bacterial enzyme. A new molecular hypothesis on the physiological regulation of oxidative phosphorylation is proposed, assuming a hormonally controlled dynamic equilibrium in vivo between two states of energy metabolism, a relaxed state with low ROS (reactive oxygen species) formation, and an excited state with elevated formation of ROS, which are known to accelerate aging and to cause degenerative diseases and cancer. The hypothesis is based on the allosteric ATP inhibition of cytochrome* c *oxidase at high intramitochondrial ATP/ADP ratios ("second mechanism of respiratory control"), which is switched on by cAMP-dependent phosphorylation and switched off by calcium-induced dephosphorylation of the enzyme.*

KEYWORDS:

cytochrome *c* oxidase · electron transport · oxidative phosphorylation · oxidoreductases · respiratory control

1. Introduction

All eukaryotic cells, under heterotrophic growth conditions, synthesize ATP in two ways: by degrading glucose to lactic acid or alcohol via glycolysis, or by oxidative phosphorylation in mitochondria. The latter pathway yields about 15 times more ATP from glucose and is essential for all multicellular organisms. The mitochondrial respiratory chain transfers reducing equivalents from nutrients successively to molecular oxygen, accompanied by storage of the released energy in a proton gradient across the inner mitochondrial membrane via three proton pumping enzyme complexes (NADH:ubiquinone oxidoreductase (I), cytochrome *c* reductase (III), and cytochrome *c* oxidase (IV)). This proton or electrochemical gradient is used by the enzyme ATP synthase for the endergonic synthesis of ATP from ADP and inorganic phosphate.[1a–c] The terminal enzyme of the respiratory chain, cytochrome *c* oxidase, reduces molecular oxygen to water without the formation of reactive oxygen species (ROS).

Cytochrome *c* oxidase from mammals is composed of 13 subunits. In addition to the three mitochondrially encoded subunits I–III, which contain the four catalytic redox centers (Cu_A, heme *a*, heme a_3, and Cu_B),[2] ten smaller subunits are nuclear-coded and partly expressed in tissue-specific isoforms.[3a–c] The yeast enzyme contains eleven,[4] that of *Dictyostelium discoideum* seven,[5] and the enzyme from the bacterium *Paracoccus denitrificans* consists of four subunits, three of which correspond to the mitochondria-encoded subunits of the eukaryotic enzyme.[6]

In previous review articles it was postulated that the increasing number of subunits during evolution reflects an increasing regulatory complexity of the enzyme.[3a,b, 7] But only within the last three to four years could regulatory functions be identified for some nuclear-coded subunits of the mammalian enzyme. These results gained strong support from the recently determined crystal structures of cytochrome *c* oxidase from *P. denitrificans*[6, 8] and bovine heart.[9–11] In the crystal structure of the bovine heart enzyme, isolated by using cholate as detergent, one of two cholate molecules was identified at a site on subunit

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VIaH (heart isoform),[10] which was previously postulated to bind ADP.[12] Although cholate and ADP have a very similar spatial structure, bound cholate, in contrast to ADP, was found to exchange with ATP very slowly[13] and prevents regulation of enzyme activity by the ATP/ADP ratio.[14]

In this Review article the current knowledge on the structure and molecular mechanism of cytochrome *c* oxidase is presented and related to new regulatory functions of subunits IV, Va, Vb, VIaH, and VIaL of the mammalian enzyme. From these results a new hypothesis on regulation of oxidative phosphorylation in vivo is proposed, based on the allosteric ATP inhibition of the enzyme through nucleotide binding to subunit IV. The allosteric inhibition by ATP at high intramitochondrial ATP/ADP ratios is hormonally regulated and suggested to prevent the increase of mitochondrial membrane potential and thus the formation of ROS. Some aspects of this hypothesis have been presented before.[15, 16]

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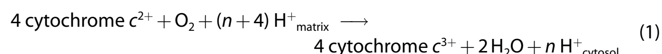
2. Crystal structures of cytochrome *c* oxidases

The crystal structure of the dimeric cytochrome *c* oxidase from bovine heart[10] is presented in Figure 1. Subunits I, II, and III, drawn in yellow, contain twelve, two, and seven transmembrane helices, respectively. The binding site for its substrate cytochrome *c*, and the Cu_A center are located in subunit II, heme *a*, and the binuclear center heme a_3/Cu_B in subunit I. Each of the ten nuclear-coded subunits are presented in different colors. Seven of them contain a single transmembrane helix each, and three are located outside the membrane: subunit VIb at the cytosolic, subunits Va and Vb at the matrix side. In the monomer on the left, the catalyzed reaction is indicated schematically [Eq. (1)].



$$4\ \text{cytochrome}\ c^{2+} + O_2 + (n+4)\ H^+_{\text{matrix}} \longrightarrow 4\ \text{cytochrome}\ c^{3+} + 2\,H_2O + n\ H^+_{\text{cytosol}} \quad (1)$$

For the monomer on the right, one binding site for 3,5-diiodothyronine, three sites for ATP or ADP binding, and a phosphorylation site at subunit I (Ser 441) are marked.

A comparison of the coordinates of the enzymes from bovine heart and *P. denitrificans*[6a,b] reveals highly conserved structures for oxygen reduction and proton translocation during evolution (see also Figure 2). In contrast, the regulatory complexity of the eukaryotic enzyme increased dramatically, as follows from the ten additional subunits present (see Sections 6 and 7).

3. The catalytic cycle

Most of the general aspects of the structure and function of cytochrome *c* oxidase discussed below refer to both the mitochondrial and the bacterial enzyme; whenever specific amino acids (or their respective mutations in the bacterial oxidase) are mentioned, the numbering system of the *P. denitrificans* enzyme is used. For a cross-reference of amino acid sequence positions in different oxidases see ref. [17].

Electrons donated from cytochrome *c* enter the oxidase complex via a conserved tryptophan in subunit II (W121; see Figure 2 and refs. [18a, b, 19a–c]), and are transferred to dioxygen through its four internal redox centers to form water. The Cu_A center, composed of two electronically coupled, mixed-valence (Cu^I/Cu^{II}) copper ions, is located at the hydrophilic domain on the cytosolic side (respectively the periplasmic side of the bacterial membrane) of subunit II. An analysis of site-directed mutants[20] has confirmed the classical view that this center is indeed the first, and only, acceptor site in the complex. The electron is subsequently transferred from the Cu_A center to heme *a* in subunit I. This low-spin heme is closer to the Cu_A center than heme a_3 (Figure 2), and therefore heme *a* is the kinetically preferred electron acceptor. Nevertheless, it was shown that in a mutant oxidase, direct electron transfer from Cu_A to heme a_3 is feasible, although with a drastically reduced efficiency.[21]

Both heme planes are oriented perpendicular to the membrane, at an angle of 108° to each other. Their iron centers are 13 Å apart, but their closest edge-to-edge distance amounts to only 5 Å; two of their histidine ligands (H411 and H413) are

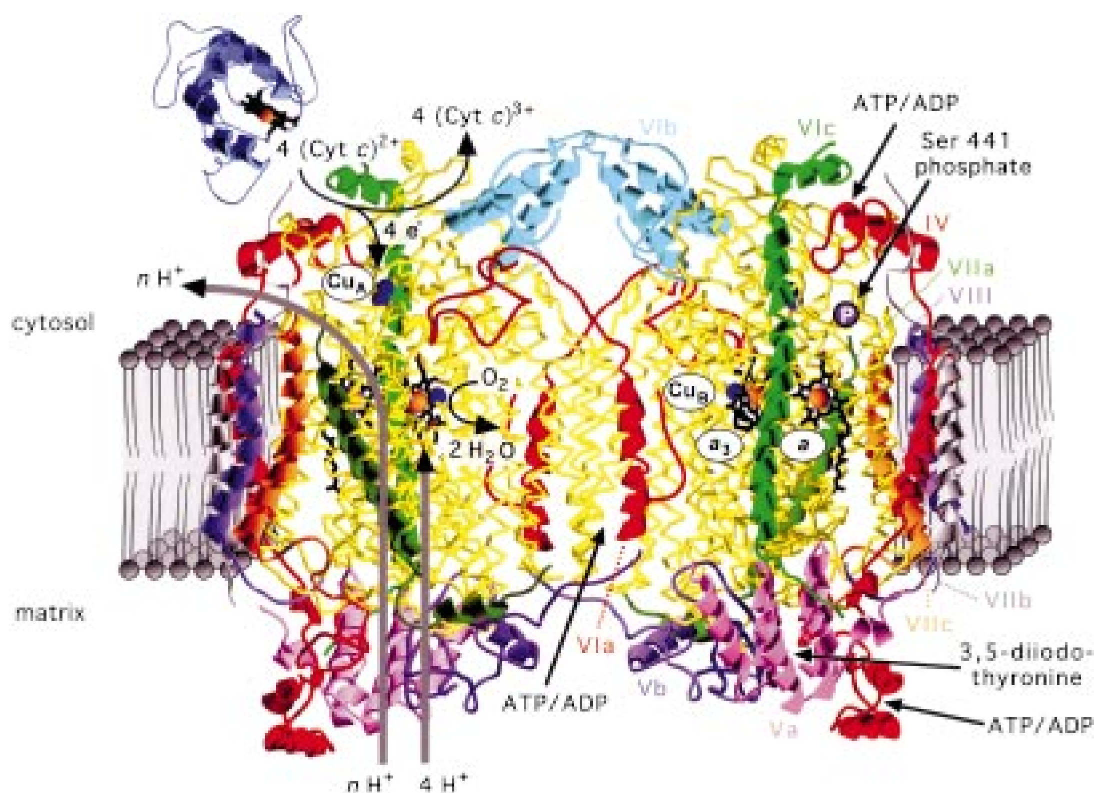


Figure 1. *Structure of the dimeric cytochrome* c *oxidase complex from bovine heart.[10] Coordinates (PDB entry 1occ) were processed with the Swiss PDB Viewer/Pov Ray program* (E. Schwan, *Ray Tracing for the Macintosh CD*, Waite Group Press, Corte Madera (CA, USA), **1994**). *The three mitochondrially coded subunits in each monomer are represented as peptide backbone traces (yellow) with their redox centers highlighted (see Figure 2 for greater detail), while the helices of nuclear-coded subunits are numbered and labeled as color-coded ribbons in the right-hand monomer. The monomer on the left schematically depicts the pathway of electrons entering from the donor, cytochrome* c *(top left drawn in blue; shown in a somewhat arbitrary position), oxygen binding to the binuclear center, and pathways and stoichiometry for matrix proton uptake and release on the cytoplasmically oriented side of the mitochondrial inner membrane (with the positioning of the phospholipid bilayer solely for illustration). In the monomer on the right, the location of the potential binding sites for ATP (or ADP) and 3,5-diiodothyronine, as well as the cAMP-dependent phosphorylation site Ⓟ on Ser 441 in subunit I are indicated (see text for details).*

spaced by only one amino acid on the same transmembrane helix X.[6] The high-spin heme a_3 and an electronically coupled Cu_B ion form the binuclear center of the enzyme (Figure 2), a motif found in most terminal oxidases.[22, 23] This center, buried about one third into the depth of the membrane, is the site of oxygen binding and water formation, requiring free access for its substrates (oxygen and protons) and probably is the key player in energy transduction (see Section 4).

The free energy of the oxidase reaction is stored in a proton gradient; it is generally agreed on that the canonical ratio of protons pumped per O_2 molecule reduction is four (but see Section 5). Unlike complex III of the respiratory chain,[23] in which a Q-cycle scheme of proton translocation is well accepted, the mechanism of proton pumping, its precise timing, and the nature of its coupling to electron transport, are still discussed in a highly controversial manner. Previous models suggested that H^+ translocation in the oxidase is strictly coupled to the oxidative part of the reaction cycle (states "P_M" to "O" in Figure 3). This view required that the first two electrons entering the binuclear center would not contribute to proton pumping at all, while the last two electrons would exhibit a microscopic pump ratio of two protons for each electron in two "power strokes".[24] In more recent sudies, this assumption has been modified,[25, 26] or dismissed altogether.[8] Closing in on the binuclear center, Figure 3 depicts current hypotheses of oxygen reduction and protonic coupling steps during one turnover. In the oxidized state ("O"), the binuclear center metal ions carry a $+3$ (heme iron) and $+2$ charge (Cu). A relevant tyrosine residue (Y 280) is present in its neutral state and forms an unusual C–N side chain cross-link to histidine 276, one of the three histidine ligands of Cu_B, in the same transmembrane helix of subunit I. This covalent bridge is observed in both crystal structures[6, 10] and confirmed by peptide sequencing.[27] Again, from the X-ray crystallographic structures it is evident that two oxygen species, possibly a water molecule and a hydroxide ion, bridge the gap between the two metal centers in the oxidized state, giving rise to the electronic coupling between both metal centers. With the first two electrons entering the binculear site, both metal centers are reduced ($+2/+1$). Current views favor the translocation of one proton already during one of these reductive steps ("E" to "R" in Figure 3; for details, see ref. [8]). It is assumed that the driving force for the pumping step emanates from a charge compensation within a hydrophobic environment.[24, 28] With two redox equivalents available at the binuclear site ("R"), oxygen is able to bind, leading to the formation of species "A" in Figure 3. On a fast time scale, this compound reacts directly to state "P_M", in which the dioxygen bond is already cleaved[29] and both oxygen atoms are present in the formal oxidation state of water.

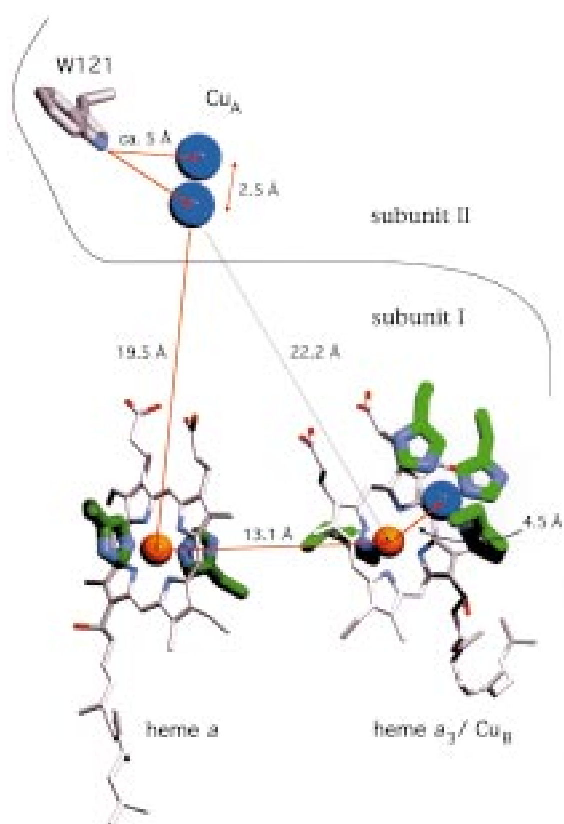


Figure 2. Redox-active metal centers in cytochrome c *oxidase subunits I and II. The low-spin heme* a *and the binuclear heme* a_3/Cu_B *center are located within the hydrophobic interior (see Figure 1) of subunit I, and are depicted with their histidine ligands. They accept electrons from* Cu_A*, a center consisting of two closely spaced copper ions located within the hydrophilic periplasmic/intermembrane domain of subunit II. W121 denotes a tryptophan acting as the presumed entry site for electrons from cytochrome* c*. Electron pathway distances are given as center-to-center distances and were calculated according to the bacterial oxidase structure.[6b] Blue spheres represent copper ions, the central Fe ions of the heme moieties are represented by orange spheres.*

How is this four-electron reduction step accounted for when only two electrons have previously reached the binuclear site? Two further electrons are provided transiently from within the site: i) the heme iron center is formally oxidized to the ferryl state (+4), and ii) the nearby cross-linked tyrosine is able to donate the fourth electron, yielding a tyrosine radical (Figure 3, P_M state highlighted). Experimental evidence in support of this reaction step has been presented recently in an EPR study.[30] The unique structural setup at the binuclear site, including the atypical side chain cross-link, seems to allow the enzyme, once oxygen is bound, to immediately split the O–O bond even if the supply of further electrons is stalled, thus avoiding the formation of reactive oxygen species such as superoxide anion or hydrogen peroxide.

The reaction scheme in the second half of the cycle (Figure 3) requires resolution of the two unusual electronic states at the binuclear center, i) reduction of the tyrosine radical by transfer of the third electron, and ii) the fourth electron to resume the oxidized state also for the iron center of heme a_3 (+3). During this last series of redox events (for details, see ref. [8]), another three proton translocation steps occur in going from the P_M to the O state. Again, each of these steps is envisaged to be "powered" by electrostatic repulsion, thus revealing the coupling between electron transport and proton translocation. Protons, previously loaded into a launch position in or above the heme propionates,[8, 21, 31] are released to the outside, an event that is triggered by the uptake of protons to the binuclear center from the inside.

4. Proton pathways in cytochrome *c* oxidase

While detailed mechanistic models exist for the coupling of proton translocation with ATP synthesis in the ATP synthase,[1a–c, 32] the mechanism of coupling electron transport with proton translocation in cytochrome *c* oxidase is still a matter of debate (see ref. [8, 26]). Wikström and co-workers were the first to demonstrate proton pumping activity of cytochrome *c* oxidase,[33a,b] but for a long time the ratio between translocated protons and transported electrons (H^+/e^- stoichiometry) remained controversial. In fact, H^+/e^- ratios between zero,[34a,b] 1.0,[35a–d] and 2.0[36a–f] were determined. After numerous discussions it was generally accepted that the H^+/e^- ratio in

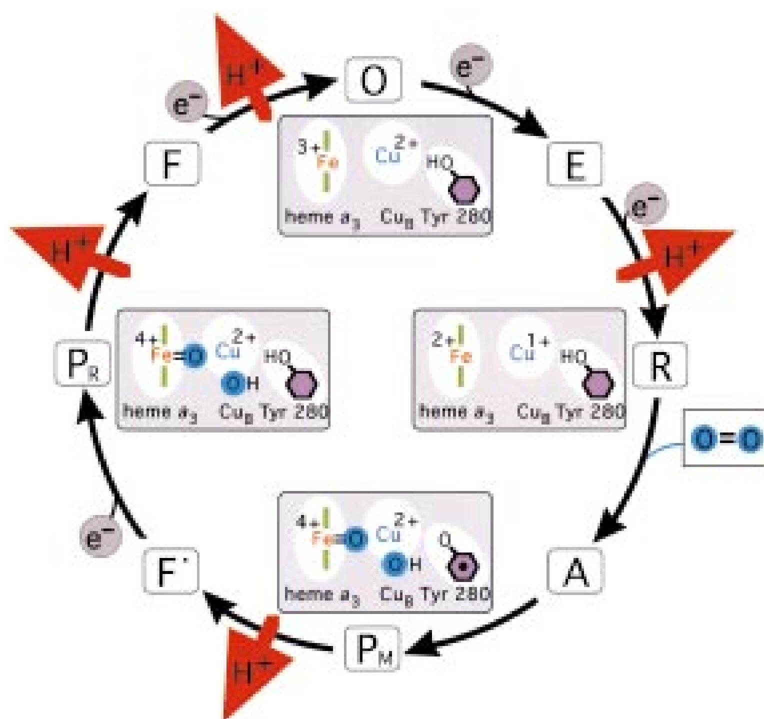


Figure 3. Simplified schematic representation of the O_2 *reduction cycle catalyzed by cytochrome* c *oxidase. Key intermediates (white boxes labeled "O", "E", "R" etc.) are listed clockwise along with the input steps for the four electrons (gray circles), the binding of dioxygen (blue), and the presumed transmembrane proton translocation steps (red arrows; according to ref. [8]). The four gray shaded boxes within the reaction circle detail the electronic states of redox components in the binuclear center of subunit I for selected redox states of cytochrome* c *oxidase, "O", "R", "*P_M*", "*P_R*"; for further explanations and references, see text. Proton uptake steps, water release, and the assignment of formal charges to the oxygen atoms have been omitted for clarity.*

cytochrome *c* oxidases of bacteria[37] and mitochondria[38] invariably is 1.0.[39a,b] The different H^+/e^- ratios found in previous studies were explained by inconsistencies in the experimental set-up.

During a complete oxygen cycle four protons are taken up from the matrix side for the formation of water ("chemical protons"), and four additional protons are assumed to be translocated from the matrix across the inner mitochondrial membrane to the cytosolic side ("pumped protons"). Theoretical considerations,[24] as well as site-directed mutagenesis studies, mainly with a structurally related quinol oxidase (see ref. [40]), suggested the existence of two different proton pathways within the hydrophobic interior of subunit I, which was supported and extended by the crystal structures of the bacterial[6] and bovine heart enzyme.[10] In the crystal structure of the bovine heart enzyme, a third proton pathway was postulated (channel "E" or "H").[10, 11] However, site-directed mutagenesis studies, performed with the bacterial enzyme, did not yield any evidence for a third pathway in the bacterial enzyme.[41a,b]

It is generally agreed (see [17]) that the K pathway, named after a crucial lysine residue (K354 in *P. denitrificans*), provides direct access for protons to the binuclear center. The D pathway, with its key residue D124 at the cytoplasmic entrance of the bacterial enzyme, extends far into subunit I and reaches another important acidic residue (E278) close to the binuclear center. The glutamate side chain may act as a gate for proton distribution.[42a,b] The further course of the D pathway, in particular its contact with the redox elements of the binuclear center and heme *a*, and its likely physical extension to the exterior side of the membrane, remain unclear at present. Early experiments seemed to indicate that the K channel is used to allow access of the four protons to the binuclear site for water formation, while the D channel was assumed to translocate protons across the membrane. From later kinetic approaches, and a separation of the reaction cycle into reductive ("eu-oxidase") and peroxidase half-reactions,[43a,b] it was concluded that the first (or the first two?) protons would pass through the K pathway, while the remaining majority would use the D pathway in a dual-purpose function, requiring some kind of distributing device. In a recent study measuring the electrogenicity of single-electron events in specific channel mutants,[44] it is concluded that the first electron reduction of the oxidized enzyme is accompanied by a compensating proton uptake through the K pathway, while the second reduction step may elicit use of the D channel. Further convincing assignments for all subsequent steps are still lacking.

With respect to the variable H^+/e^- ratio (see Section 5) it is interesting to note that several mutations in presumed residues lining the D channel lead to a partial decoupling of electron transport from proton translocation. In one case, a single mutation in an asparagine residue (N131 →D), located above the canonical D-channel entrance residue D124, yielded an ideally uncoupled phenotype.[45] After reconstitution in liposomes, proton pumping of the mutated enzyme was completely abolished, while electron transport under turnover conditions proceeded at rates identical to that of the wild-type enzyme.

5. Variable H^+/e^- stoichiometries in cytochrome *c* oxidase

Several recent observations indicate that the H^+/e^- stoichiometry in cytochrome *c* oxidase may indeed be variable, and this variation is not dependent on the measuring conditions. For a bacterial cytochrome *c* oxidase, cytochrome ba_3 oxidase from *Thermus thermophilus*, a H^+/e^- ratio of 0.5 instead of 1.0 was measured under standard conditions.[46] Decreased H^+/e^- ratios in mammalian cytochrome *c* oxidase are obtained after removal of subunit III (for reviews see ref. [47, 48]), and after chemical modification with reagents specific for carboxy groups in hydrophobic environments like DCCD (dicyclohexylcarbodiimide)[47, 48] and EEDQ (*N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline).[49]

For rat liver mitochondria, Murphy and Brand[50a,b] investigated the charge stoichiometry ($q^+/2e^-$) over a wide range of membrane potentials $\Delta\Psi_m$ (120 – 180 mV), both for cytochrome *c* oxidase and cytochrome *c* reductase (cytochrome bc_1 complex). They found no change in stoichiometry for cytochrome *c* reductase, but a decrease in $q^+/2e^-$ ratio from 4 at low $\Delta\Psi_m$ to 2 at high $\Delta\Psi_m$ for cytochrome *c* oxidase, corresponding to a decrease in H^+/e^- pump ratio from 1 to 0. Similar results were obtained by Papa and co-workers, who measured the H^+/e^- ratio in the presence of the K^+ ionophore valinomycin, which converts $\Delta\Psi_m$ into ΔpH_m. With isolated mitochondria[51] as well as with the reconstituted enzyme[51b–c] a decrease in H^+/e^- ratio with increasing flow rate, that is, increasing pH gradient, was found for cytochrome *c* oxidase, but not for cytochrome *c* reductase.

6. High ATP/ADP ratios decrease the H^+/e^- stoichiometry only in the heart

A decrease of the H^+/e^- ratio from 1.0 to 0.5 with increasing intraliposomal ATP/ADP ratio was measured with reconstituted cytochrome *c* oxidase from bovine heart.[52a,b] The decrease was half-maximal at an ATP/ADP ratio of 100 (for the free nucleotides), and correlated to the exchange of bound ADP by ATP at the matrix domain of subunit VIaH, since preincubation of the enzyme with a monoclonal antibody against subunit VIaH prevented the decrease.[12, 52a] It should be mentioned that only approximately 5% of total cellular ADP represents "free ADP", due to its binding to a large number of intracellular sites.[53] In rat heart, based on ^{31}P NMR data, cytosolic ATP/ADP ratios of 100 – 1000 have been estimated,[54a] contrasting the much lower values for ATP/ADP ratios when the total nucleotide content is taken into account.[54b] Considering the electrogenic nature of the ADP/ATP carrier, the intramitochondrial ATP/ADP ratios for the free nucleotides would be about 20 – 200.

The nucleotide-binding site at subunit VIaH was verified in the crystal structure of the enzyme by identification of a cholate molecule at this site, since cholate is structurally very similar to ADP.[10] Numerous attempts to crystallize the bovine heart enzyme in the presence of ATP or ADP have been unsuccessful.[55] This could be due to the large number of nucleotide-binding sites—seven for ATP or ADP and three additional ones

only for ADP in the bovine heart enzyme, as identified by equilibrium dialysis.[14, 56] In the crystallized enzyme ten tightly bound cholate molecules have been identified,[14] which could stabilize the enzyme in a rigid conformation. In the presence of nucleotides the enzyme could exist in various conformational states, thus preventing crystallization.

A decrease in H^+/e^- ratio from 0.9 (presence of ADP) to 0.3 by ATP was also described for cytochrome *c* oxidase from a cyanobacterium (*Synechocystis* sp. PCC6803) containing a fourth subunit homologous to the eukaryotic subunit IV.[57] With reconstituted cytochrome *c* oxidase from bovine liver (and kidney) a H^+/e^- ratio of 0.5 was measured under the same conditions as the ones applied to the bovine heart enzyme. The enzymes from heart or skeletal muscle (VIaH) and from liver or kidney (VIaL) contain different isoforms of subunit VIa (and subunits VIIa and VIII). The lower H^+/e^- ratio of the liver enzyme was neither influenced by the intraliposomal ATP/ADP ratio nor by a monoclonal antibody against subunit VIaH (and subunit VIc).[52b] To our knowledge, the H^+/e^- stoichiometry of the liver-type enzyme was so far only measured in isolated mitochondria, which showed H^+/e^- ratios between 1 and 2 (see Section 5). It remains to be investigated whether in mitochondria the enzyme exists in a different state (e.g. phosphorylated), resulting in higher H^+/e^- ratios.

Interestingly, in liver, heart, and skeletal muscle of a bird (turkey) only the liver-type isoform (VIaL) was found, and the reconstituted enzymes from turkey liver and heart showed H^+/e^- ratios of 0.5, which were independent of the intraliposomal ATP/ADP ratio.[58] It was postulated that the decreased H^+/e^- ratio for the enzyme from skeletal muscle tissue (subunit VIaH) at high ATP/ADP ratios (e.g. during sleep), and for the enzyme from nonskeletal muscle tissues, participate in thermogenesis.[52b, 59]

In fish (trout, carp, and tuna) a third type of subunit VIa was identified, which differs equally from the mammalian isoforms VIaH and VIaL.[58, 60] This can be clearly seen in the most parsimonious phylogram of Figure 4, describing the evolutionary relationships between cytochrome *c* oxidase subunits VIa from different species. It is possible that cytochrome *c* oxidase from fish permanently exhibits a high H^+/e^- ratio in order to keep thermogenesis low.

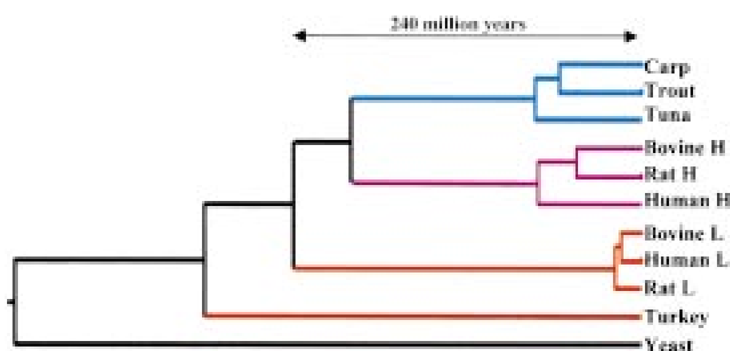


Figure 4. Most parsimonious phylogram describing the evolutionary relationship between cytochrome c *oxidase subunits VIa. The comparisons were done with mature amino acid sequences using the Meg Align 1993–97 program with the Jotun–Hein algorithm.[61] The evolution of three different types of subunit VIa in animals is evident from the figure.*

7. Two different mechanisms of "respiratory control"

"Respiratory control" was originally defined as stimulation of oxygen uptake of isolated mitochondria after addition of ADP (active, state 3 respiration) and subsequent inhibition due to transformation of ADP into ATP (controlled, state 4 respiration).[62a, b] This phenomenon could be explained by the chemiosmotic hypothesis:[63] The three proton pumps of the respiratory chain (NADH:ubiquinone oxidoreductase, cytochrome *c* reductase, and cytochrome *c* oxidase) generate the proton motive force Δp [Eqs. (2) and (3); $Z = 2.303\,RT/F$], which is used by ATP synthase for the synthesis of ATP from ADP and inorganic phosphate.

$$\Delta p = \Delta \Psi_m - Z\Delta pH_m \quad (2)$$

$$\Delta p F = \Delta \mu_{H^+} \quad (3)$$

Stimulation of ATP synthase by ADP, taken up into mitochondria by the ATP/ADP carrier, decreases Δp and stimulates mitochondrial respiration. After conversion of ADP into ATP, Δp increases again and respiration becomes inhibited, because proton pumps are inhibited at high Δp values ("first mechanism of respiratory control").

Recently a "second mechanism of respiratory control" was found, which is independent of Δp and based on the inhibition of cytochrome *c* oxidase activity at high intramitochondrial ATP/ADP ratios, changing the kinetics from hyperbolic to sigmoidal behavior (activity/[cytochrome *c*] or *v*/[S] relationship).[64a, b] The nucleotides bind to the matrix domain of the transmembraneous subunit IV. This was concluded from preincubation of the enzyme with a monoclonal antibody against subunit IV, which prevented the allosteric ATP inhibition, and from the requirement of high ATP/ADP ratios inside the vesicles for the inhibition of the ascorbate respiration of mitochondrial particles.[64a] Based on the crystal structure of the bovine heart enzyme,[10] the binding site for ATP (or ADP) on the matrix domain of subunit IV was predicted from molecular modeling studies. It could be located in a pocket close to the membrane formed by amino acids from subunits IV, II, and I.[64c] Half-maximal inhibition of activity is obtained at an intramitochondrial ATP/ADP ratio of 28,[64d] which is lower than the normal ratio of 100, decreasing the H^+/e^- stoichiometry of the bovine heart enzyme to half-maximal extent.[52a] The ATP/ADP ratio of 28 lies within the expected intramitochondrial ratio of free nucleotides in vivo (see Section 6). The maximal Hill coefficient of 2[64a] indicates cooperativity of two cytochrome *c* binding sites, each of which is assumed to be located at one monomer of the dimeric enzyme complex. An allosteric ATP inhibition was also found in the yeast enzyme, but not in the three-subunit enzyme from *Rhodobacter sphaeroides*,[65] which, like most bacteria, lacks the homologous subunit IV. An exception in this respect is cytochrome *c* oxidase from cyanobacteria (*Synechocystis* sp. PCC6803) which contains a fourth subunit that is homologous to the

eukaryotic subunit IV. The cyanobacterial enzyme is also regulated by ATP.[57]

A modification of the allosteric ATP inhibition is possible by further binding of ATP to the cytosolic side of subunit IV (Figure 1), which increases the K_M value for cytochrome *c*.[13] Furthermore, a cAMP-independent phosphorylation of subunit IV (see Section 8) was described by Steenaart and Shore,[66] but its effect on the enzymatic activity is unknown. Recently a second isoform of subunit IV (IV-2) was identified in tuna fish, showing only 50% identity to subunit IV-1.[67] The two isoforms of subunit IV in animals correspond to the homologous two isoforms of subunit V in yeast (Va and Vb),[68a] which are exclusively expressed under normoxic (subunit Va), or suboxic conditions (subunit Vb).[68b] Based on immunological data, an isoform of subunit IV has been previously postulated to be expressed in mammalian fetal tissue[69a] or skeletal muscle.[69b] Meanwhile, the isoform of subunit IV (IV-2) has been characterized for the rat, mouse, and human enzymes.[64c]

The allosteric inhibition of cytochrome *c* oxidase by ATP is sensitive to several experimental and physiological parameters that "uncouple" the second mechanism of respiratory control, so that high ATP/ADP ratios do no longer induce allosteric inhibition of enzyme activity. These parameters include the use of dodecylmaltoside as detergent, which monomerizes the enzyme,[64a] TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine), which is frequently used as an electron carrier between ascorbate and cytochrome *c*, micromolar concentrations of 3,5-diiodothyronine, which binds specifically to the matrix-oriented subunit Va,[70a] as well as submicromolar concentrations of palmitate.[70b] In addition, the allosteric inhibition by ATP is critically dependent on sufficient amounts of cardiolipin in the soluble as well as in the reconstituted enzyme.[64a]

8. Hormones control the "second mechanism of respiratory control"

As mentioned in Section 7, 3,5-diiodothyronine (T_2) acts directly on cytochrome *c* oxidase and abolishes the allosteric ATP inhibition by binding to subunit Va, as verified by binding of radioactively labeled T_2 and suppression of its effect by preincubation of the enzyme with a monoclonal antibody against subunit Va.[70a] Subunit Va is located adjacent to the ATP-binding site of subunit IV (Figure 1). The effect of T_2 could explain the long known "short-term effect" of thyroid hormones on the resting metabolic rate.[71]

Hormones, acting through cAMP as second messenger, switch on the allosteric ATP inhibition by cAMP-dependent phosphorylation of cytochrome *c* oxidase, and hormones increasing the intracellular Ca^{2+} concentration switch it off by calcium-induced dephosphorylation. This was concluded from experiments with the isolated enzyme from bovine heart and with mitochondria from bovine liver.[15, 72] In isolated mitochondria the allosteric inhibition of cytochrome *c* oxidase by ATP is generally not observed, probably due to dephosphorylation or to bound palmitate. In contrast, incubation of bovine liver mitochondria with cAMP in the presence of ATP induces the allosteric inhibition by ATP (via mitochondrial protein kinases), which is strongly amplified by the presence of KF,[15] an unspecific inhibitor of protein phosphatases. The site of cAMP-dependent phosphorylation was determined for bovine heart cytochrome *c* oxidase by incubation with protein kinase A, cAMP, and [γ-^{32}P]ATP. The autoradiography showed labeling of subunits I, II (or III), and Vb.[72] In the bovine heart enzyme, consensus sequences for cAMP-dependent phosphorylation[73] only occur in subunits I (RRYS^{441}, cytosolic side), III (RES^{65}, matrix side), and Vb (RCPS^{84}, matrix side). Interestingly, the consensus sequence in subunit I is found in man, cow, frog, fly and sea urchin, that is, in animals controlled by hormones, but not in primrose, yeast and bacteria.[74a] In order to identify the membrane side where phosphorylation induces inhibition of activity in the presence of ATP, the reconstituted bovine heart enzyme was either phosphorylated only from the inside (matrix side) in a cAMP-dependent manner, resulting in decreased activity with hyperbolic kinetics, or from the outside (cytosolic side), resulting in decreased activity with sigmoidal kinetics[74b]. These results suggest that the allosteric inhibition of cytochrome *c* oxidase by ATP at high intramitochondrial ATP/ADP ratios (through exchange of bound ADP by ATP at the matrix domain of subunit IV) is switched on by cAMP-dependent phosphorylation of Ser 441 in subunit I by a mitochondrial protein kinase located in the intermembrane space.

Recently, the specific binding of the regulatory subunit of protein kinase A (RIα) to subunit Vb of cytochrome *c* oxidase was shown in HeLa cells.[75] Since subunit Vb is located exclusively in the matrix (Figure 1), a protein kinase A must also occur within mitochondria. This conclusion is supported by results of Scacco et al.,[76] who found stimulation of NADH dehydrogenase activity in fibroblast cultures after cAMP-dependent phosphorylation of the matrix-oriented 18-kDa subunit.

9. A new molecular-physiological hypothesis

We propose a new molecular hypothesis on the physiological regulation of oxidative phosphorylation in vivo. We postulate that in cells a hormonally controlled dynamic equilibrium exists between two states of energy metabolism: a relaxed state with efficient oxidative phosphorylation according to ATP utilization, and associated with low $\Delta\Psi_m$ and low ROS formation, and an excited state with increased $\Delta\Psi_m$, but less efficient oxidative phosphorylation, associated with increased ROS formation. The relaxed state is switched on by cAMP-dependent phosphorylation of cytochrome *c* oxidase at Ser 441 in subunit I (and possibly of subunits III and Vb), and switched off by calcium ion induced dephosphorylation (Figure 5).

In isolated mitochondria high membrane potentials of 150 – 200 mV are usually measured.[63, 77] In contrast, low $\Delta\Psi_m$ values of 100 – 150 mV were determined in perfused hearts.[78] We suggest that in vivo cytochrome *c* oxidase is mostly (in a cAMP-dependent manner) phosphorylated, and respiration is mainly controlled (except for control by substrates) by the intramitochondrial ATP/ADP ratio (second mechanism of respiratory control). This mechanism converts cytochrome *c* oxidation into the rate-limiting step of the respiratory chain, which was verified in vivo by metabolic control analysis in intact cells.[79a–c] In mitochondria,

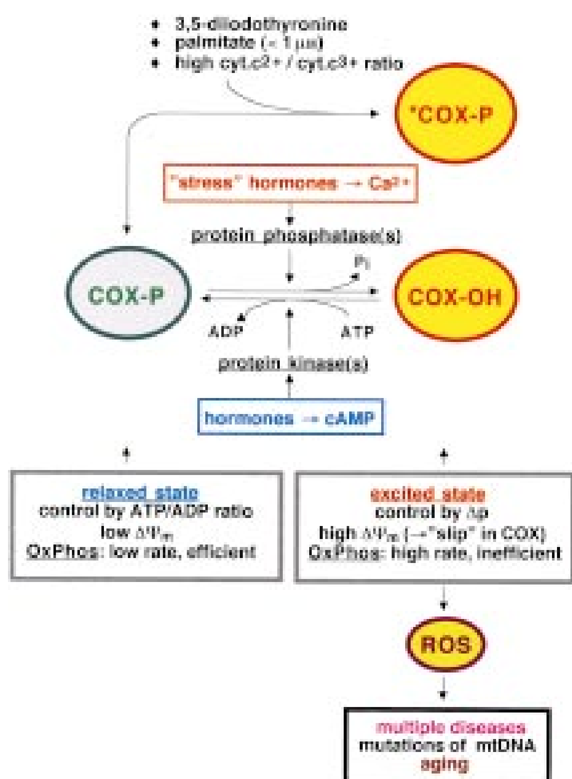


*Figure 5. Schematic representation of the postulated regulation of oxidative phosphorylation (OxPhos) in vivo. We postulate that in eukaryotic cells a hormonally controlled dynamic equilibrium exists between two states of energy metabolism: 1) A relaxed state with efficient OxPhos according to the utilization of ATP at low proton motive force Δp (mainly $\Delta\Psi_m = 100-150$ mV) and low ROS formation. This state is switched on by cAMP-dependent phosphorylation of the enzyme (COX-P). 2) An excited state with increased but less efficient OxPhos at high Δp (150 – 200 mV) and enhanced ROS formation. The increased Δp causes a decrease in H^+/e^- ratio ("slip") in cytochrome c oxidase and increases the rate of respiration and ATP synthesis due to decreased efficiency ($-\Delta G^{o'}$ is more negative). The excited state is switched on i) by dephosphorylation of cytochrome c oxidase (COX-OH) induced by "stress" hormones through Ca^{2+}-activated mitochondrial protein phosphatase(s), ii) without dephosphorylation (*COX-P) by binding 3,5-diiodothyronine or free palmitate; or by increased substrate pressure in the mitochondrial respiratory chain (high cyt.c^{2+}/cyt.c^{3+} ratio) as a consequence of excess substrate supply. The excited state stimulates the formation of ROS, which are considered the main cause of aging (via mutations of mitochondrial DNA, mtDNA), degenerative diseases, and cancer.*

the allosteric inhibition of cytochrome *c* oxidase by ATP stabilizes low values of the proton motive force Δp (mainly $\Delta\Psi_m$), due to feedback inhibition by high ATP/ADP ratios, as shown schematically in Figure 6. The simultaneous control of the activity of the Δp consumer (ATP synthase) and the Δp generator (proton pumps) by the intramitochondrial ATP/ADP ratio keeps $\Delta\Psi_m$ low, since high ATP/ADP ratios are already obtained at $\Delta\Psi_m$ values of 100 – 120 mV. This follows from results of Kaim and Dimroth,[80] who determined the $\Delta\Psi_m$ dependence of the rate of ATP synthesis by ATP synthase and obtained saturation and maximal rates at 100 – 120 mV.

In the absence of the allosteric inhibition of cytochrome *c* oxidase by ATP, cell respiration is limited by Δp (mainly $\Delta\Psi_m$, in addition to substrate control), which inhibits the proton pumps at high values (150 – 200 mV). In isolated mitochondria[63, 77] and with the reconstituted enzyme,[49, 81] high values of $\Delta\Psi_m$ are usually measured, probably due to dephosphorylation of cytochrome *c* oxidase. We propose that in vivo "stress hormones" lead to an increase in $\Delta\Psi_m$ (150 – 200 mV) through calcium-induced dephosphorylation of cytochrome *c* oxidase, and thus turn on the excited state of energy metabolism. The dephosphorylated enzyme no longer represents the rate-limiting step of the respiratory chain, as measured by metabolic control analysis with isolated mitochondria, showing a five- to tenfold excess capacity of cytochrome *c* oxidase.[82a, b]

The allosteric inhibition of cytochrome *c* oxidase by ATP (with subsequent increase in $\Delta\Psi_m$) is also released without dephosphorylation by 3,5-diiodothyronine,[68] free palmitate (< 1 µM),[70b] and high substrate concentrations (high cytochrome c^{2+}/cytochrome c^{3+} ratios, as a consequence of excess nutritional substrates) due to the sigmoidal *v*/[S] kinetics.[64a] At high $\Delta\Psi_m$ values the H^+/e^- ratio of cytochrome *c* oxidase decreases (see Section 5.). It was proposed that the physiological role of hormone-induced dephosphorylation of cytochrome *c* oxidase is to increase the driving force (Δp) and thus the rate of ATP synthesis under stress at the expense of the efficiency of oxidative phosphorylation[15, 16] (see Figure 5).

High values of $\Delta\Psi_m$, however, have deleterious consequences for the whole organism. Investigations of Liu[83a, b] and Korshunov et al.[83c] have shown that with increasing $\Delta\Psi_m$ values (above 150 mV) the formation of ROS in mitochondria increases. The production of ROS is assumed to occur in the respiratory chain by direct one-electron transfer from ubisemiquinone to dioxy-

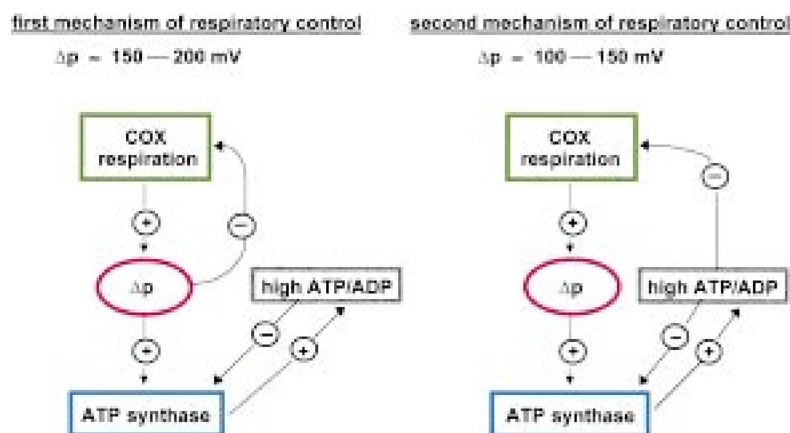


Figure 6. Control of mitochondrial proton motive force Δp (mainly $\Delta\Psi_m$) by the rates of respiration (COX activity) and ATP synthase. The scheme compares the control of cell respiration (according to the classical chemiosmotic hypothesis) by the proton motive force Δp (first mechanism of respiratory control, left panel) and by the intramitochondrial ATP/ADP ratio (second mechanism of respiratory control, right panel), which, at high ATP/ADP ratios, leads to allosteric inhibition of cytochrome c oxidase (COX) activity by ATP, and thus of mitochondrial respiration. This feedback inhibition of respiration at high intramitochondrial ATP/ADP ratios stabilizes Δp at low values (100 – 150 mV) that are sufficient for the synthesis of ATP by ATP synthase. After hormonal switching off the second mechanism of respiratory control, Δp rises because now mitochondrial respiration (except for control by substrate supply) is only controlled by high values of Δp (150 – 200 mV).[63]

gen.[84] ROS cause mutations of mitochondrial DNA,[85a,b] which are assumed to represent the main contributor to aging[86a–d] and degenerative diseases.[87] In addition, ROS have been suggested to cause cancer.[88]

10. Observations supporting the postulated control of $\Delta\Psi_m$ and ROS formation

Robb-Gaspers et al.,[89a,b] using fluorescent dyes, simultaneously measured cytosolic and/or mitochondrial calcium ion concentration ($[Ca^{2+}]_c$ and $[Ca^{2+}]_m$, respectively) and mitochondrial $\Delta\Psi_m$ or ΔpH_m after addition of vasopressin or thapsigargin to cultivated hepatocytes. The immediate increase in $[Ca^{2+}]_c$ (saturation after about 50 s) was followed by a slow but sustained increase in both $\Delta\Psi_m$ and ΔpH_m. This result contrasts the decrease in $\Delta\Psi_m$ after addition of Ca^{2+} to isolated mitochondria, but is explained by Ca^{2+}-activated dephosphorylation of cytochrome *c* oxidase, abolition of allosteric inhibition by ATP, and consequent stimulation of Δp.

In exercise physiology, the "O_2 drift" phenomenon has been known for more than 25 years but remained unexplained. As exercise work loads are increased, oxygen consumption increases linearly until a sufficiently high work load is reached at which O_2 consumption increases disproportionately, and "drifts" upward rather then reaching a steady state.[90a–c] This phenomenon can be explained by a switch at high work load from the relaxed to the excited state of energy metabolism through Ca^{2+}-activated dephosphorylation of cytochrome *c* oxidase.

The neurotoxic effect of glutamate or *N*-methyl-D-aspartate (NMDA) was related to increased formation of ROS in mitochondria. By using a specific fluorescent dye (DCF-H2) the increase in ROS formation was measured in neurons after addition of glutamate. The effect was essentially dependent on activation of the NMDA receptor and the presence of Ca^{2+} in the medium.[91a] Corresponding experiments by Dugan et al.[91b] with cultivated cortical neurons of mice, using dihydrorhodamin 123 as ROS indicator, demonstrated also increased ROS formation after addition of NMDA, which was dependent on the presence of extracellular Ca^{2+}.

Electron paramagnetic resonance (EPR) measurements of oxygen radicals in skeletal muscle samples from human, rat, and mouse after excessive contractile activity indicated increased ROS formation.[92] The authors postulated that muscle damage after excessive muscle activity is based on the increased formation of free radicals.

The comparison of ROS formation in different mammals and birds showed that animals with maximal life expectancy are characterized by low mitochondrial ROS formation, and this is independent of the specific resting metabolic rate of individual animals.[93]

11. Summary and outlook

We have presented the current view on the catalytic mechanism of cytochrome *c* oxidase, based on the crystal structures of the bacterial and bovine heart enzymes, and on site-directed mutagenesis studies with bacterial enzymes. The surprisingly similar crystal structures of the catalytic center of the two enzymes contrasts the large difference in the number of their subunits, 3 – 4 in bacteria and 13 in mammals. For some nuclear-coded subunits of the eukaryotic enzyme, which are absent in bacteria, regulatory functions could be elucidated.

A "second mechanism of respiratory control" was found, which connects respiration in mammalian cells to the consumption of ATP by inhibiting cytochrome *c* oxidase activity at high intramitochondrial ATP/ADP ratios through the exchange of bound ADP by ATP at the matrix domain of subunit IV. This allosteric inhibition of the eukaryotic enzyme by ATP is hormonally switched on by cAMP-dependent phosphorylation and switched off by Ca^{2+}-activated dephosphorylation, and also by low concentrations of 3,5-diiodothyronine or palmitate. A decrease in the proton pumping stoichiometry of the bovine heart enzyme at high ATP/ADP ratios was found through binding of ATP to subunit VIaH. This decrease in energy transduction efficiency, not found in the bovine liver enzyme (containing subunit VIaL), is assumed to participate in mammalian thermogenesis.

Based on these results a new molecular hypothesis on the physiological regulation of oxidative phosphorylation is postulated, suggesting in vivo a hormonally controlled dynamic equilibrium between two states of energy metabolism: a relaxed state with efficient oxidative phosphorylation at low mitochondrial membrane potential and low ROS formation, and a "stress-induced" excited state with increased but less efficient oxidative phosphorylation at high membrane potential and elevated ROS formation. The excited state is suggested to accelerate aging and to cause degenerative diseases and cancer.

In this review some regulatory functions of cytochrome *c* oxidase subunits IV, Va, Vb, VIaH, and VIaL have been described. The functions of subunits VIb, VIc, VIIa (two isoforms) VIIb, VIIc, and VIII (two isoforms) are still unknown. Also, from the seven identified high-affinity binding sites for ATP or ADP the function of only two could be elucidated (at subunits IV and VIaH). We are only at the beginning of understanding the extensive regulatory complexity of eukaryotic cytochrome *c* oxidase.

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