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Regulation of oxidative phosphorylation, the mitochondrial membrane potential, and their role in human disease

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Abstract Thirty years after Peter Mitchell was awarded the Nobel Prize for the chemiosmotic hypothesis, which links the mitochondrial membrane potential generated by the proton pumps of the electron transport chain to ATP production by ATP synthase, the molecular players involved once again attract attention. This is so because medical research increasingly recognizes mitochondrial dysfunction as a major factor in the pathology of numerous human diseases, including diabetes, cancer, neurodegenerative diseases, and ischemia reperfusion injury. We propose a model linking mitochondrial oxidative phosphorylation (OxPhos) to human disease, through a lack of energy, excessive free radical production, or a combination of both. We discuss the regulation of OxPhos by cell signaling pathways as a main regulatory mechanism in higher organisms, which in turn determines the magnitude of the mitochondrial membrane potential: if too low, ATP production cannot meet demand, and if too high, free radicals are produced. This model is presented in light of the recently emerging understanding of mechanisms that regulate mammalian cytochrome *c* oxidase and its substrate cytochrome *c* as representative enzymes for the entire OxPhos system.

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Introduction

The mitochondrial oxidative phosphorylation (OxPhos) process utilizes electrons predominantly delivered by NADH, and also by $FADH_2$, derived from food or energy stores of the organism. These electrons are transferred via the electron transport chain (ETC) to the final electron acceptor oxygen, which is reduced to water. The ETC is an assembly line consisting of NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), ubiquinone, bc_1-complex (complex III), cytochrome *c* (Cyt *c*), and cytochrome *c* oxidase (CcO; complex IV). Electron transport in the ETC is coupled to the proton pumping activity of complexes I, III, and IV, which generates the mitochondrial proton motive force Δp_m across the inner mitochondrial membrane. Δp_m is utilized by ATP synthase (complex V) to synthesize ATP from ADP and phosphate, which is coupled to the backflow of protons from the mitochondrial intermembrane space to the matrix. OxPhos is the main driving force of all cellular processes since it provides more than 15 times the ATP generated by anaerobic glycolysis (Alberts et al. 2002). It was Peter Mitchell who proposed that an electrochemical gradient utilized to produce ATP is the mechanism underlying OxPhos (Mitchell 1961). Mitchell's chemiosmotic hypothesis has withstood scientific scrutiny and is one of the main scientific accomplishments of the twentieth century and beyond.

Ensuing research then focused on elucidating structural and mechanistic processes of individual OxPhos complexes at the molecular level. Much progress has been made to crystallize the OxPhos complexes from bacteria and eukaryotes in the presence or absence of substrates, substrate analogs, and inhibitors. Complete structures have been published for complexes II (e.g., from pig heart; Sun et al. 2005), III (e.g., from cow heart; Iwata et al. 1998), IV (e.g., from *Paracoccus denitrificans* (Ostermeier et al. 1995) and from cow heart (Tsukihara et al. 1996)), and Cyt *c* (e.g., from horse heart; Sanishvili et al. 1995), and partial structures are available for complex I (for the hydrophilic matrix arm from *Thermus thermophilus*; Sazanov and Hinchliffe 2006) and ATP synthase (e.g., the F_O unit from *Escherichia coli*; Rastogi and Girvin 1999), the F_1 unit from cow heart (Gibbons et al. 2000) and liver (Chen et al. 2006), and parts of the peripheral stalk from cow heart (Dickson et al. 2006). The structural information is priceless and has made possible the development and testing of models related to electron transfer within the complexes and its coupling to the proton pumping mechanisms, which is still not fully understood.

Regulatory mechanisms acting on OxPhos

This review focuses on mammalian CcO and Cyt *c*, for which regulation has been studied in more detail compared to the other OxPhos complexes, and which shall therefore serve as representative proteins of the entire OxPhos system. The reaction of Cyt *c* and CcO comprises the last step of the ETC. Reduced Cyt *c* transfers electrons to CcO that are passed down to molecular oxygen, which is reduced to water (Fig. 2). The latter reaction requires protons that are taken up from the matrix, which also contribute to the generation of the mitochondrial membrane potential, in addition to protons that are pumped by CcO.

We will first discuss known regulatory mechanisms acting on OxPhos. Then we will present a model of the regulation of the mitochondrial membrane potential $\Delta\Psi_m$ with potentially far-reaching implications for human health: $\Delta\Psi_m$ is directly linked to ATP production but also to free radical production, and a lack of energy and/or excessive free radical production are involved in most human diseases.

Basic regulation of OxPhos: substrates and the reaction intermediate Δp_m

In addition to providing mechanistic insights, structural information continues to provide a better understanding of regulatory mechanisms of OxPhos. The main regulatory mechanisms that act on OxPhos are summarized in Fig. 1 and include availability of substrates such as NADH, ADP and phosphate, and the proton motive force Δp_m as the reaction intermediate that inhibits the proton pumps at high values. This basic regulation plays the central role in bacteria. In higher organisms additional features evolved to fine tune OxPhos function, which can be readily seen in the increased complexity of OxPhos components. For example, CcO from bacteria contains two to four subunits whereas the mammalian enzyme is composed of 13 subunits per monomer and functions as a dimer (Tsukihara et al. 1996).

OxPhos supercomplexes and substrate channeling

In addition to dimers of OxPhos complexes, respiratory supercomplexes or respirasomes have been reported in eukaryotes with various stoichiometries including I–III_2, I–III_2–IV, I–III_2–IV_2, I–III_2–IV_3, I–III_2–IV_4 (for current reviews see Boekema and Braun 2007; Lenaz and Genova 2006; Schägger 2002). The arrangement of OxPhos components into assembly lines would make possible increased electron transfer rates due to substrate channeling as was shown in vitro for the electron transfer between complexes I and III (Schägger and Pfeiffer 2000). A separate supercomplex organization was found for ATP synthase with the phosphate and ADP/ATP carriers (Chen et al. 2004a), allowing efficient delivery and removal of substrates and products of ATP synthesis. If supercomplex formation can be regulated through cell signaling pathways it could allow increased energy production when needed, such as during exercise. On this note, it was recently shown for ATP synthase, which can occur in monomeric and higher order forms, that the γ subunit was tyrosine

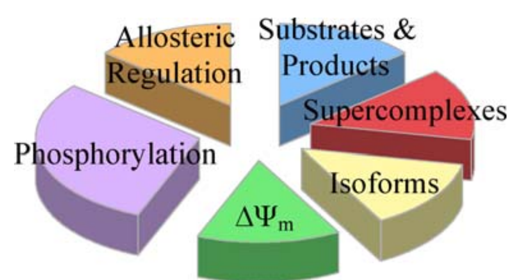

Fig. 1 Main regulatory mechanisms acting on OxPhos complexes. Bacterial OxPhos is mainly regulated by substrate availability and the membrane potential. Higher eukaryotic organisms have developed additional regulatory mechanisms, including the expression of tissue-specific isoforms, allosteric regulation, and most importantly, as we propose, reversible phosphorylation: this regulation allows controlled respiration and avoids high mitochondrial membrane potential $\Delta\Psi_m$ levels, a condition known to yield excessive radical production. Additional regulatory mechanisms, such as other posttranscriptional modifications (e.g., acetylation or oxidation of methionines; Schilling et al. 2006) or competitive inhibition of CcO by nitric oxide, are not shown

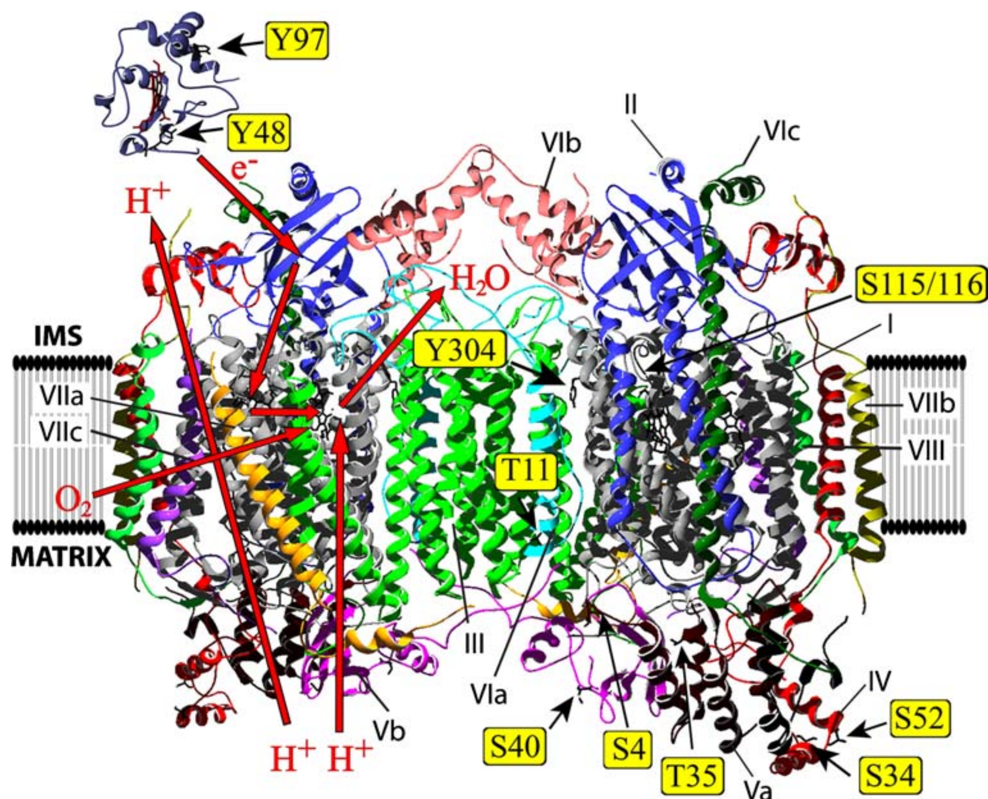

Fig. 2 Mapped phosphorylation sites of mammalian cytochrome *c* oxidase and cytochrome *c*. Crystallographic data of cow heart CcO (Tsukihara et al. 1996) and horse heart Cyt *c* (Sanishvili et al. 1995) were processed with the program Swiss-PDBViewer 3.7 and rendered using the program POV-Ray 3.6. Identified mammalian phosphorylated amino acids are highlighted for Cyt *c*, and on the right monomer of the CcO dimer (stick representation). On the left monomer the electron flow from reduced Cyt *c* via Cu_A, heme *a*, heme a_3/Cu_B, and molecular oxygen, which is coupled to proton pumping activity of CcO, is schematically illustrated (*red arrows*). Identified phosphorylation sites are: Y97 from cow heart Cyt *c* (Lee et al. 2006); Y48 from cow liver Cyt *c* (Yu et al. 2008); T11 of CcO subunit VIa from cow heart (Tsukihara et al. 2003); Y304 of CcO subunit I from cow liver (Lee et al. 2005); S115 and S116 of CcO subunit I, T52 of subunit IV, and S40 of subunit Vb in rabbit heart (Fang et al. 2007); and S34 of CcO subunit I and S4 and T35 of subunit Va from cow heart (Helling et al. 2008)

phosphorylated only in the monomeric but not in the dimeric state (Di Pancrazio et al. 2006).

Regulatory adaptation at the tissue level—isoform expression

Another common regulatory mechanism involves the expression of tissue-specific and developmentally regulated isoforms. Such isoforms are known for Cyt *c* and CcO, which are encoded by separate genes. Cyt *c* is expressed as a somatic and a testes-specific isoform in most mammals, such as rodents (Hennig 1975), whereas the syntenic region of testes Cyt *c* in the human genome contains a non-transcribed pseudogene (Hüttemann et al. 2003). Mouse testes-specific Cyt *c* shows distinct functional properties compared to the somatic isoform with a threefold increased activity to reduce hydrogen peroxide and a three to fivefold higher activity to induce apoptosis (Liu et al. 2006).

CcO contains three liver- and heart-type isoform pairs of subunits VIa, VIIa, and VIII, a lung-specific isoform of CcO subunit IV, a testes-specific isoform of subunit VIb, and a third isoform of subunit VIII. Functional data have been reported for the liver- and heart-type isozymes although there are major discrepancies among individual studies, which may be explained by the fact that phosphorylation was not considered and not maintained during mitochondria and CcO isolation (reviewed in Hüttemann et al. 2007b). Generally, liver-type CcO, which is expressed in tissues like brain with a lower mitochondrial density, shows a higher activity compared to the heart-type isozyme, which is found in heart and skeletal muscle tissue with a high mitochondrial capacity (Vijayasarathy et al. 1998). Lung CcO shows an about 2.5 fold increased activity compared to liver CcO (Hüttemann et al. 2007a), and the presence of the lung isoform results in increased capability to dissipate H_2O_2 buildup (Fukuda et al. 2007). It has therefore been proposed that by increasing electron flux through the ETC

fewer electrons are available for ROS formation, which might be particularly important in the highly oxygenated lung tissue, which faces the highest levels of the other ingredient for ROS formation—oxygen (Hüttemann et al. 2007a).

Allosteric regulation

Both Cyt *c* and CcO share a regulatory mechanism via adenine nucleotides, which results in an inhibition of respiration in the presence of ATP, a condition when cells are at rest and do not require increased OxPhos activity. Binding of ATP to Cyt *c* leads to an inhibition of the reaction between Cyt *c* and CcO and the elimination of the low K_m phase of the otherwise biphasic kinetics with CcO (Ferguson-Miller et al. 1976). The ATP binding site was mapped to the conventional "left side" of the molecule (McIntosh et al. 1996; Fig. 2, top left).

A similar 'energy rheostat' regulatory mechanism is found in CcO. It is mediated by an ATP/ADP binding pocket located in the matrix domain of the largest nuclear-encoded subunit (IV), leading to allosteric inhibition of CcO in the presence of ATP and activation in the presence of ADP (Arnold and Kadenbach 1999; Hüttemann et al. 2001; Napiwotzki et al. 1997). For the discussion of other possible allosteric control mechanisms acting on CcO including divalent cation binding sites, fatty acids, and the thyroid hormone diiodothyronine, see Hüttemann et al. (2007b).

Competitive inhibition of CcO via nitric oxide

The physiological and pathological role of nitric oxide (NO) on mitochondrial function is a rapidly expanding research field, which can only be briefly touched on here (for reviews see Brown 2001; Giulivi 2007). NO synthesis is catalyzed by four types of NO synthase (NOS), a neuronal (nNOS) and an endothelial NOS (eNOS) isoform that are constitutively expressed, an inducible form (iNOS), and mitochondrial NOS (mtNOS). mtNOS can directly interact with CcO on the matrix side by binding to subunit Va (Persichini et al. 2005), which increases local concentrations of NO near its target enzyme. Mechanistically, NO competes with oxygen at the binuclear heme a_3–Cu_B binding site, which leads to reversible CcO inhibition (Brown and Cooper 1994; Giulivi 1998). Increased cellular NO levels coincide with many human diseases, including arthritis (Cuzzocrea 2006), sepsis and septic shock (Assreuy 2006), cancer (Fukumura et al. 2006), obesity, and diabetes (Stepp 2006). In addition, we have recently shown that traumatic brain injury (TBI) leads to a strong induction of NO production, concomitantly decreased CcO activity, and more than 60% decreased ATP levels analyzed at 4 and 24 h post-TBI (Hüttemann et al. 2008). CcO activities were partially restored by application of iNOS antisense oligonucleotides to downregulate iNOS expression, leading to a full normalization of ATP levels similar to the controls. A lack of energy after TBI caused by inhibition of CcO was proposed as an important aspect of trauma pathology (Hüttemann et al. 2008). The inhibitory effect of NO on cell respiration has also been proposed as a general mechanism underlying neurodegenerative diseases through over-production of NO (Moncada and Bolanos 2006).

In contrast, a beneficial application of NO is currently discussed as a cardioprotective therapy for ischemia/reperfusion injury due to its direct effects on mitochondria (Burwell and Brookes 2008). Based on our model discussed in greater detail below, which links regulation of OxPhos complexes with both energy and free radical production, inhibition of the proton pumps prior to or during reperfusion makes sense. This is so because cellular stress in the form of ischemia would lead to dephosphorylation and maximal activation of the ETC complexes, followed by a hyper-polarization of the mitochondrial membrane potential, a condition known to produce excessive amounts of free radicals. Thus, inhibition of the terminal step of the ETC may be capable of preventing the otherwise inevitable excessive production of ROS and cell death.

Cell signaling pathways

It is now evident that all mammalian OxPhos components including Cyt *c* are targeted for phosphorylation (for a recent detailed discussion of those phosphorylations based on the crystal structures and kinase predictions, see Hüttemann et al. 2007b). To date, 17 phospho-epitopes have been mapped, mainly by tandem-mass spectrometry analysis. The corresponding signaling pathways are known for only a few of the sites mapped, and the kinases and phosphatases catalyzing these reversible phosphorylations remain unknown (or uncertain). In addition to phosphorylation sites on Cyt *c* and CcO that are discussed below, the following sites have been identified on complexes I and V: complex I from cow heart was phosphorylated on S95 of the B14.5a subunit (Pocsfalvi et al. 2006), on S59 of the 42 kDa subunit (Pocsfalvi et al. 2006; Schilling et al. 2005), and on S20 of the ESSS and S55 of the MWFE subunits (Chen et al. 2004b); complex V was phosphorylated on T213 (T163 of the mature peptide) of the β-subunit in human skeletal muscle (Hojlund et al. 2003), S76 (S33 of the mature peptide) of the α-subunit of mouse brain, and Y75 of the δ-subunit in human NIH3T3 cells (Ko et al. 2002).

Phosphorylation of cytochrome *c*

The 12 kDa small and highly positively charged electron carrier Cyt *c* is located in the mitochondrial intermembrane space and transfers electrons from bc_1 complex to CcO. The fact that Cyt *c* is targeted by cell signaling pathways has only been revealed very recently. As detailed below, we were able to demonstrate that the in vivo phosphorylation state of Cyt *c* is distinct between liver and heart tissue, but that it is the phosphorylated form that was found in both cases. Importantly, preservation of protein phosphorylation during protein isolation is critical for these studies and can be achieved by the inclusion of unspecific serine/threonine phosphatase inhibitor KF (10 mM), unspecific tyrosine phosphatase inhibitor vanadate (1 mM), and calcium chelator EGTA (2 mM) to prevent activation of calcium-dependent protein phosphatases. Detailed protocols are now available (Lee et al. 2006; Yu et al. 2008). Older protocols omitted at least one of these compounds, in particular vanadate, which turns out to be crucial since tyrosine phosphorylation is the modification found in Cyt *c*.

Isolation of cow heart Cyt *c* followed by mass spectrometry revealed phosphorylation on tyrosine 97 (Lee et al. 2006; Fig. 2). This phosphorylation produced pronounced sigmoidal kinetics and thus inhibition in the reaction with CcO. Half-maximal turnover was obtained at Cyt *c* substrate concentrations of 5.5 and 2.5 μM for Y97-phosphorylated and alkaline phosphatase-treated dephosphorylated Cyt *c*. Y97 is not part of the predicted Cyt *c*-CcO binding site (Roberts and Pique 1999), but its phosphorylation leads to spectral changes of the weak 695 nm absorption band. This absorption is caused by the methionine-heme iron bond, indicating that a phosphorylation event on the periphery of the molecule affects the heme environment.

The discovery of a second phosphorylation on Cyt *c* was a surprise. Using cow liver tissue instead of heart we observed Y48-phosphorylation after analysis by mass spectrometry (Yu et al. 2008). This tyrosine is located in the lower frontal region of Cyt *c* (Fig. 2). It is near to the predicted Cyt *c*-CcO binding site and the closest residue on CcO within 4 Å is K58 of the nuclear encoded subunit VIIa. Although Y48 points inward (Fig. 2), the addition of the bulky and negatively charged phosphate group might lead to a rotation of the residue, perhaps allowing salt bridge formation with nearby K58 and changing the on/off rates for the Cyt *c*-CcO interaction. We analyzed the in vitro reaction kinetics with isolated CcO from cow liver and found strong differences. Maximal turnover of Tyr-48 phosphorylated Cyt *c* was 3.7 s^{-1} whereas dephosphorylation resulted in a 2.2 fold increase in activity to 8.2 s^{-1}.

Regulation of Cyt *c* by phosphorylation raises the interesting question of the effect on the other functions of Cyt *c* that still have to be addressed, particularly as a radical scavenger under normal conditions (Pereverzev et al. 2003), and during apoptosis, which centrally involves the release of Cyt *c* from the mitochondria and subsequent binding to Apaf1. The release of Cyt *c* is preceded by its action as a lipid peroxidase during early apoptotic stages, where it selectively oxidizes the mitochondrial lipid cardiolipin to which Cyt *c* is attached (Kagan et al. 2005). Interestingly, the peroxidase mechanism involves a Cyt *c* tyrosine radical (Tyurina et al. 2006), and one may speculate that phosphorylation of the corresponding tyrosine residue may prevent this reaction, thus protecting the cell from undergoing apoptosis. However, in addition to Y48 and Y97 there are two additional conserved tyrosine candidates.

Phosphorylation of cytochrome *c* oxidase

In an initial study, incubation of rat heart mitochondria with [γ-^{32}P]ATP suggested phosphorylation of CcO subunit IV by an endogenous kinase (Steenaart and Shore 1997). As in other studies, the identification of the phosphorylation site and possible functional effects were not addressed. Other studies based on in vitro approaches used purified CcO in combination with a commercially available kinase (e.g., Bender and Kadenbach 2000). Although such experiments showed the possibility of modification, its physiological significance has to be questioned, because cellular structures and auxiliary proteins that mediate specificity are not present (discussed in Hüttemann et al. 2007b). Earlier, our group had tested a variety of kinases in an in vitro context but dismissed this approach due to numerous unspecific signals on various CcO subunits that were not obtained using an in vivo approach.

We therefore attempted to address the effect of signaling pathways on CcO function in a more physiological context by using tissue and intact cell culture cells. Using liver tissue we investigated the cAMP-dependent pathway, which functions as a starvation signal in this tissue and can be selectively increased with starvation hormone glucagon (Ainscow and Brand 1999). Our study of cow liver tissue revealed that high cAMP levels lead to Y304 phosphorylation of CcO catalytic subunit I (Fig. 2), as was demonstrated by mass spectrometry (Lee et al. 2005), suggesting that a downstream tyrosine kinase is activated by the cAMP pathway. Phosphorylated CcO was almost completely inhibited up to 10 μM Cyt *c* substrate concentrations (Lee et al. 2005). Other groups have studied mitochondrial

function in a cAMP-dependent context in heart tissue (e.g., Technikova-Dobrova et al. 2001); however, it is noteworthy that in heart there is no such specific physiological trigger to raise intracellular cAMP, and G-protein linked receptor-mediated increase of cAMP is often accompanied by an increase in calcium. Apparently, signaling pathways can operate differently in a tissue-specific manner.

Another example of CcO inhibition through phosphorylation is ischemic stress as was shown with rabbit heart leading to phosphorylation of S115 and S116 of subunit I, T52 of subunit IV, and S40 of subunit Vb (Fang et al. 2007; Prabu et al. 2006; Fig. 2).

Other identified phosphorylation sites in cow heart CcO without any special treatment are T11 of subunit VIa based on the crystal structure (Tsukihara et al. 2003), and S34 of subunit IV and S4 and T35 of subunit Va based on high quality mass spectrometry analysis (Helling et al. 2008; Fig. 2).

In addition to the above identified phosphorylation sites, there is evidence for more signaling acting on CcO. Non-receptor tyrosine kinase c-Src was shown to phosphorylate subunit II in osteoblasts, a process that appears to be important for the bone-resorbing activity of these cells, and which might be altered in rheumatoid arthritis (Miyazaki et al. 2003, 2006). This phosphorylation activates CcO. Based on in silico prediction, Y113 of subunit II might be the target of c-Src phosphorylation. It is predicted as a possible phosphorylation site by NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) and this site produces a medium stringency hit for c-Src using the kinase prediction tool Scansite (http://scansite.mit.edu/motifscan_seq.phtml). In addition this site is fully accessible and located on the tip of subunit II.

Hypoxic stress signaling during ischemia involves protein kinase C (PKC) signaling. Although several PKC isoforms appear to be present in mitochondria to some extent, the PKCε isoform seems to be specifically enriched in the mitochondrial fraction after ischemia (Guo et al. 2007). PKCε was found to interact with CcO subunit IV by co-immunoprecipitation, and an increase in CcO activity was reported after stimulation of the signaling pathway (Ogbi et al. 2004; Ogbi and Johnson 2006). Yet another example of translocation of a kinase to mitochondria followed by interaction with CcO, in this case subunit II, was reported for the epidermal growth factor receptor (EGFR), which was shown by phage display screening and co-immunoprecipitation after activation of the pathway (Boerner et al. 2004). Phosphorylation of CcO by PKCε and EGFR and the identification of the precise sites remain to be addressed, preferably in vivo under conditions preserving phosphorylation as described above.

The regulation of the mitochondrial membrane potential, energy and free radical production, and their implications for human disease

Concomitantly with electron flow from substrates to oxygen, complexes I and III in addition to CcO pump protons from the mitochondrial matrix to the intermembrane space creating an electrochemical proton gradient across the highly proton-impermeable inner mitochondrial membrane. This electrochemical gradient or protonmotive force (Δp_m) consists of two components, an electrical component or the mitochondrial membrane potential ($\Delta\Psi_m$), and the chemical component or the pH difference across the inner mitochondrial membrane. Their interdependance is defined by the equation $\Delta p_m = \Delta\Psi_m - 59\Delta pH$. The electrical component represents the major portion of Δp_m, and can be monitored in absolute scale, for example by measurement of the distribution of a membrane permeable cation such as tetraphenylphosphonium (TPP) with a TPP electrode using isolated mitochondria. More frequently, relative changes of $\Delta\Psi_m$ in living cells are reported using positively charged voltage dependent fluorescent probes that accumulate in the mitochondria dependent upon the magnitude of $\Delta\Psi_m$.

Studies on isolated mitochondria imply that $\Delta\Psi_m$ can be influenced by electrogenic processes (e.g., ATP/ADP exchange, Ca^{2+} accumulation), but also by electroneutral processes when ΔpH and $\Delta\Psi_m$ are interconverted. For example, transport of inorganic phosphate into mitochondria decreases ΔpH via $[P_i + H^+]_{intermembrane\ space} \rightarrow [P_i + H^+]_{matrix}$; however, this decrease is compensated by a subsequent increase of $\Delta\Psi_m$ through increased activity of the proton pumps (Lambert and Brand 2004; Nicholls 1974).

In the intact cell, the build-up of Δp_m is in part counterbalanced by its utilization for ATP synthesis, and for other processes such as protein import into mitochondria, metabolite transport, and ion cycling. In the traditional view, Δp_m regulates the activity of the ETC complexes: at high potentials further proton pumping is inhibited, whereas a decrease of Δp_m through proton utilization, e.g., by ATP synthase, would in turn allow the ETC to rebuild Δp_m. Thus, mitochondria in intact cells respire between the extreme energetic states, state 3 (in the presence of ADP) and state 4 (when ADP has been converted into ATP), as defined by studies on isolated mitochondria. Due to the difficulty of measuring absolute Δp_m values in intact cells the following discussion is based on the numerous values reported for $\Delta\Psi_m$, which constitutes the major part of Δp_m.

Studies based on isolated mitochondria or reconstituted CcO vesicles in general showed high $\Delta\Psi_m$ values ≥150 mV often exceeding 200 mV (Nicholls and Ferguson 1992; Steverding and Kadenbach 1991). Reported $\Delta\Psi_m$

values for frequently studied liver mitochondria under state 4 conditions are 150 mV (Nicholls 1974), 172 mV (Labajova et al. 2006), 196 mV (Cossarizza et al. 1996; Barger et al. 2003), 202 mV (Shears and Kirk 1984), 200–210 mV (Brand et al. 1988), and 210–220 mV (da Silva et al. 1998), and isolated brain mitochondria showed $\Delta\Psi_m$ values around 190 mV (Moreira et al. 2001).

Studies performed in a more physiological context using intact cells or even intact organs generally revealed lower values in the range of 80–160 mV. For example, 100–140 mV were observed in perfused rat hearts depending on workload and substrates (Wan et al. 1993), 105 mV and 81 mV were reported for intact cultured fibroblasts and neuroblastoma cells, respectively (Zhang et al. 2001), 120 mV for lymphocytes (Brand and Felber 1984), 117 mV for embryonic heart cells (Backus et al. 1993), 134 mV for 143B osteosarcoma cells (Porteous et al. 1998), 150 mV for neurons (Nicholls 2006), and values between 143 and 161 mV for hepatocytes (Cortese 1999; Hoek et al. 1980; Nobes et al. 1990). In addition, $\Delta\Psi_m$ is influenced by age and decreases over time: hepatocytes from young rats showed a $\Delta\Psi_m$ value of 154 mV whereas cells from 20–28 month old rats revealed three defined subpopulations of cells with $\Delta\Psi_m$ values of 70 mV, which was the largest population, in addition to 93 mV, and 154 mV (Hagen et al. 1997).

Differences between reported $\Delta\Psi_m$ values from different species and tissues may in part be explained by their distinct mitochondrial functions and protein composition, including the presence of tissue specific isoforms, which may alter OxPhos functionality. Reported $\Delta\Psi_m$ value differences from the same species and tissue may in part be accounted for by experimental conditions. This may be particularly important when analyzing isolated mitochondria, and one might argue that non-optimal buffer composition (e.g., K^+ concentration) and the mitochondria-to-measuring-buffer ratio might affect measurements. In addition, state 3 and state 4 respiration are extreme experimental conditions for isolated mitochondria, whereas intact cells operate somewhere in between. However, $\Delta\Psi_m$ values reported for isolated mitochondria during state 3, when ATP synthase utilizes protons leading to a decrease of $\Delta\Psi_m$, e.g., 172 mV for rat liver mitochondria (Cossarizza et al. 1996), often exceed those values observed for intact cells, which arguably represent a more state 4-like situation when analyzed under standard, non-stressed conditions. Thus, there appears to be a discrepancy between the higher values observed in isolated mitochondria compared to intact cells, which at first sight might not be easily explained.

An obvious question comes to mind, whether eukaryotic cells rely on $\Delta\Psi_m$ regulation through ATP turnover, i.e., respiratory control, or rather recruit other mechanisms, such as reversible phosphorylation, to control the electron

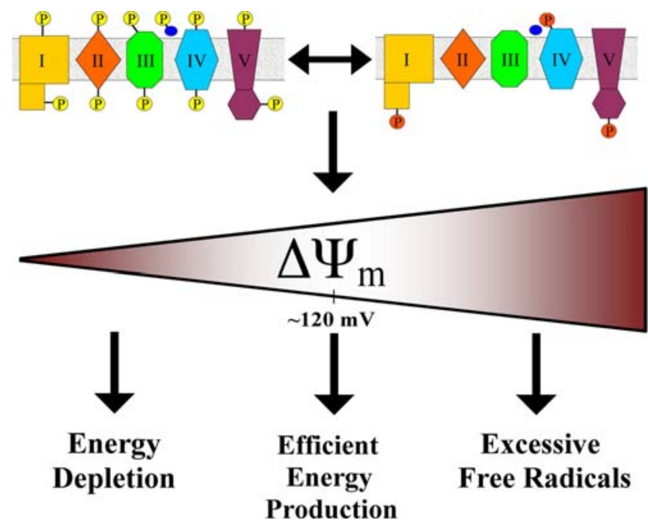

Fig. 3 Hypothesis that the mitochondrial membrane potential in higher organisms is largely controlled through the regulation of the activity of the OxPhos complexes via reversible phosphorylation. In the model there are three distinct $\Delta\Psi_m$ zones, (a) partially depolarized with insufficient protons for ATP synthase to maintain energy homeostasis, (b) an optimal "healthy" $\Delta\Psi_m$ range around 100–140 mV that allows sufficient energy production but avoids the production of free radicals, and (c) $\Delta\Psi_m$ values >150 mV that do not further accelerate ATP production, but are known to lead to the production of free radicals, which increases exponentially at higher $\Delta\Psi_m$ values. Examples of known signaling components that lead to a change in the phosphorylation state of OxPhos complexes are Ca^{2+} (dephosphorylation) and cAMP and Src signaling (phosphorylation). Under healthy conditions OxPhos complexes are found in various phosphorylated forms that control their activity. In particular the proton pumps (complexes *I*, *III*, and *IV*) are structurally changed such that $\Delta\Psi_m$ values ≥150 mV inhibit further proton pumping, preventing higher $\Delta\Psi_m$ levels and concomitant free radical production. This controlled state also allows allosteric regulation of CcO by the ATP/ADP ratio, a built-in energy rheostat. However, under stressed conditions, the proton pumps are mainly dephosphorylated and maximally active, and only $\Delta\Psi_m$ levels of ≥200 mV are now inhibitory. This leads to free radical production but does not increase ATP production, because ATP synthase already works at maximal capacity at healthy $\Delta\Psi_m$ levels of about 120 mV. However, the intrinsic capability of OxPhos to generate such high $\Delta\Psi_m$ levels is important, e.g., during induction of type II apoptosis, which is accompanied by a transient hyperpolarization of $\Delta\Psi_m$ and thus production of free radicals that may be a key signal for commitment to cell death

and proton fluxes in and across the inner mitochondrial membrane. We propose that the latter is the case in higher organisms and that dephosphorylation of proteins during mitochondria isolation may explain higher $\Delta\Psi_m$ values compared to the in vivo state. In this model (Fig. 3) OxPhos complexes occur in a variety of phosphorylation states that control their activity. In particular, complexes I, III, and IV, which pump protons, are structurally changed such that $\Delta\Psi_m$ values ≥150 mV inhibit further proton pumping, preventing higher $\Delta\Psi_m$ levels. In contrast, the dephosphorylated complexes are maximally active and $\Delta\Psi_m$ only

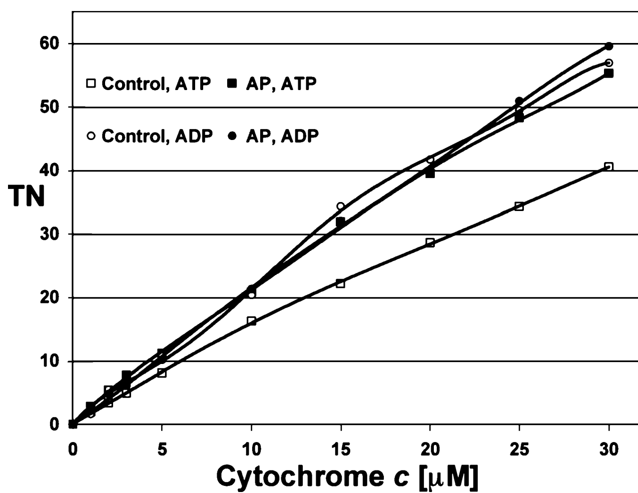

Fig. 4 Phosphorylation of cytochrome c oxidase enables allosteric regulation by the ATP/ADP ratio. CcO was treated with alkaline phosphatase (AP, from cow intestine, Roche) at 10 °C for 24 h. Control CcO was incubated under identicals condition in the absence of AP. CcO was dialyzed with cardiolipin and ATP overnight at 4 °C. CcO activity was analyzed in a closed 200 μl chamber equipped with a micro Clark-type oxygen electrode (Oxygraph system, Hansatech) in the presence of 5 mM ATP (*closed symbols*) after incubation in the presence of the pyruvate kinase/phosphoenolpyruvate ATP regenerating system (Lee et al. 2005). For measurements in the presence of 5 mM ADP (*open symbols*), incubation was performed in the absence of pyruvate kinase. Turnover number (TN) is defined as oxygen consumed (μmol)/[min·CcO (μmol)]. Note that this assay was performed in the absence of two problematic reagents that affect enzyme kinetics: the detergent dodecylmaltoside, which was suggested to monomerize CcO (Suarez et al. 1984), and *N,N,N′,N′*-tetramethyl-p-phenylendiamine (TMPD), which facilitates electron transfer and as a result Cyt *c* does not have to dissociate from CcO in order to be re-reduced (Ferguson-Miller et al. 1976)

inhibits further proton pumping at very high values, with potentially deleterious consequences as discussed below.

Based on studies of CcO and Cyt *c* the in vivo form of both enzymes is the phosphorylated form, which can be modulated by signaling pathways. For example, phosphorylation of CcO subunit I Y304 and both tyrosine phosphorylations on Cyt *c* lead to inhibited—or better, "controlled"—respiration, a principle that may also apply to the other OxPhos complexes. In addition, there appears to be a hierarchical order of the regulation of CcO in the sequence *control by phosphorylation* > *allosteric control*: dephosphorylation of CcO with unspecific alkaline phosphatase (AP) abolishes allosteric regulation through the ATP/ADP ratio (Fig. 4, closed symbols). CcO used in this experiment was tyrosine phosphorylated on subunit IV (and II) prior to AP treatment (Lee et al. 2005). Since subunit IV contains the ATP/ADP binding site as discussed earlier, this regulatory mechanism might be enabled when this subunit is phosphorylated.

It is our hypothesis that mitochondria under normal conditions in intact cells do not work at full capacity (Fig. 3). However, there are circumstances where the latter may well occur in vivo, and calcium is a likely key player in this process. It is the most important signal for mitochondrial activation (Robb-Gaspers et al. 1998), and it was recently shown in a proteomic study to induce dephosphorylation of most mitochondrial proteins (Hopper et al. 2006), likely mediated through calcium-dependent mitochondrial phosphatases as we and others have proposed (Bender and Kadenbach 2000; Hüttemann et al. 2007b; Lee et al. 2005).

Importantly, dephosphorylation of mitochondrial proteins also occurs during mitochondria isolation if no precautions are taken, such as the inclusion of phosphatase inhibitors, which is crucial based on our experience, but used by only very few laboratories. During mitochondria isolation cell structures are broken and cell signaling from the plasma membrane and the cytoplasm to the mitochondria is no longer possible. However, phosphatases retained in the mitochondria will continuously dephosphorylate mitochondrial proteins. This will produce highly active OxPhos enzymes operating at maximal capacity, and according to our model explains the high $\Delta\Psi_m$ values found for isolated mitochondria. These higher $\Delta\Psi_m$ values of >150 mV may not translate to increased $\Delta\Psi_m$ utilization via ATP production because maximal rates of ATP synthesis by ATP synthase occur at $\Delta\Psi_m = 100 - 120\text{mV}$ (Kaim and Dimroth 1999).

Since complex regulatory mechanisms to control OxPhos by reversible phosphorylation may result in lower $\Delta\Psi_m$ values, why did they evolve in higher organisms? First, excess capacity of OxPhos is desirable, because mitochondria have to adapt to varying energy demands. However, we propose that the major rationale for lower membrane potentials is to avoid free radical production because $\Delta\Psi_m$ is directly related to the production of ROS: at higher potentials starting at $\Delta\Psi_m$>140 mV, ROS production increases exponentially (Korshunov et al. 1997; Liu 1999), whereas mitochondria of resting cells with a low mitochondrial membrane potential $\Delta\Psi_m$ do not produce significant amounts of ROS (Liu 1999). Thus, the maintenance of lower, physiologically optimal $\Delta\Psi_m$ values avoids the generation of ROS but provides the full capability to produce ATP at $\Delta\Psi_m = 100 - 120\text{mV}$ (Kaim and Dimroth 1999). In addition, the control of OxPhos also makes possible its participation in mitochondrial (type II) apoptosis: after the induction of apoptosis $\Delta\Psi_m$ has been repeatedly observed to increase, leading to a transient hyperpolarization and increased free radical production, which has been proposed as a key signal for committing a cell to apoptosis (reviewed in Kadenbach et al. 2004). Bacteria lack those elaborate regulatory means, because selection is based on proliferation rate instead of the fine-tuned metabolic control required for cell survival and

communication between cells and organs in higher organisms. Equipping bacteria with a complex regulatory system would be a growth disadvantage, but is indispensable in multicellular organisms.

Conclusion

The model presented has several implications for human health, among them that disease conditions involving OxPhos and their possible future therapies should be viewed in light of two key contributing factors, a lack of energy and/or increased free radical production. For example, free radicals have been linked to many human diseases: in addition to aging and cancer, free radicals are involved in Alzheimer's disease, the spread of AIDS between cells, atherosclerosis, and alcoholism (Cahill et al. 2002; Dreher and Junod 1996; Hoozemans et al. 2002; Kameoka et al. 1993; Olinski et al. 2002; Ozawa 1997). In addition, many of the pathological effects of diabetes have been proposed to result from increased production of free radicals by the respiratory chain during hyperglycemia (Baynes 1991; Green et al. 2004; Wolff 1993), and decreasing the mitochondrial membrane potential has been proposed as a target for diabetes therapy (Green et al. 2004). Therefore, a research priority should be the identification of phosphorylation sites and signaling molecules that comprise the underlying regulatory mechanisms. This will make possible the elucidation of structure-function relationships and subsequently modulation of mitochondrial function. Advances in technologies used to identify posttranslational modifications can be expected to allow quantitation of protein phosphorylation in the future, and enable researchers to distinguish between background and physiologically relevant signals. Thus, studying mitochondrial proteins as terminal targets of signal transduction pathways holds tremendous potential for better understanding and treating human diseases.

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References

Ainscow EK, Brand MD (1999) The responses of rat hepatocytes to glucagon and adrenaline. Application of quantified elasticity analysis. Eur J Biochem 265:1043–1055

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2002) The molecular biology of the cell. Garland Science, New York

Arnold S, Kadenbach B (1999) The intramitochondrial ATP/ADP-ratio controls cytochrome c oxidase activity allosterically. FEBS Lett 443:105–108

Assreuy J (2006) Nitric oxide and cardiovascular dysfunction in sepsis. Endocr Metab Immune Disord Drug Targets 6:165–173

Backus M, Piwnica-Worms D, Hockett D, Kronauge J, Lieberman M, Ingram P, LeFurgey A (1993) Microprobe analysis of Tc-MIBI in heart cells: calculation of mitochondrial membrane potential. Am J Physiol 265:C178–C187

Barger JL, Brand MD, Barnes BM, Boyer BB (2003) Tissue-specific depression of mitochondrial proton leak and substrate oxidation in hibernating arctic ground squirrels. Am J Physiol Regul Integr Comp Physiol 284:R1306–R1313

Baynes JW (1991) Role of oxidative stress in development of complications in diabetes. Diabetes 40:405–412

Bender E, Kadenbach B (2000) The allosteric ATP-inhibition of cytochrome c oxidase activity is reversibly switched on by cAMP-dependent phosphorylation. FEBS Lett 466:130–134

Boekema EJ, Braun HP (2007) Supramolecular structure of the mitochondrial oxidative phosphorylation system. J Biol Chem 282:1–4

Boerner JL, Demory ML, Silva C, Parsons SJ (2004) Phosphorylation of Y845 on the epidermal growth factor receptor mediates binding to the mitochondrial protein cytochrome c oxidase subunit II. Mol Cell Biol 24:7059–7071

Brand MD, Felber SM (1984) Membrane potential of mitochondria in intact lymphocytes during early mitogenic stimulation. Biochem J 217:453–459

Brand MD, Hafner RP, Brown GC (1988) Control of respiration in non-phosphorylating mitochondria is shared between the proton leak and the respiratory chain. Biochem J 255:535–539

Brown GC (2001) Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase. Biochim Biophys Acta 1504:46–57

Brown GC, Cooper CE (1994) Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. FEBS Lett 356:295–298

Burwell LS, Brookes PS (2008) Mitochondria as a target for the cardioprotective effects of nitric oxide in ischemia-reperfusion injury. Antioxid Redox Signal 10:579–599

Cahill A, Cunningham CC, Adachi M, Ishii H, Bailey SM, Fromenty B, Davies A (2002) Effects of alcohol and oxidative stress on liver pathology: the role of the mitochondrion. Alcohol Clin Exp Res 26:907–915

Chen C, Ko Y, Delannoy M, Ludtke SJ, Chiu W, Pedersen PL (2004a) Mitochondrial ATP synthasome: three-dimensional structure by electron microscopy of the ATP synthase in complex formation with carriers for Pi and ADP/ATP. J Biol Chem 279:31761–31768

Chen R, Fearnley IM, Peak-Chew SY, Walker JE (2004b) The phosphorylation of subunits of complex I from bovine heart mitochondria. J Biol Chem 279:26036–26045

Chen C, Saxena AK, Simcoke WN, Garboczi DN, Pedersen PL, Ko YH (2006) Mitochondrial ATP synthase. Crystal structure of the catalytic F1 unit in a vanadate-induced transition-like state and implications for mechanism. J Biol Chem 281:13777–13783

Cortese JD (1999) Rat liver GTP-binding proteins mediate changes in mitochondrial membrane potential and organelle fusion. Am J Physiol 276:C611–C620

Cossarizza A, Ceccarelli D, Masini A (1996) Functional heterogeneity of an isolated mitochondrial population revealed by cytofluorometric analysis at the single organelle level. Exp Cell Res 222:84–94

Cuzzocrea S (2006) Role of nitric oxide and reactive oxygen species in arthritis. Curr Pharm Des 12:3551–3570

da Silva EM, Soares AM, Moreno AJ (1998) The use of the mitochondrial transmembrane electric potential as an effective biosensor in ecotoxicological research. Chemosphere 36:2375–2390

Di Pancrazio F, Bisetto E, Alverdi V, Mavelli I, Esposito G, Lippe G (2006) Differential steady-state tyrosine phosphorylation of two oligomeric forms of mitochondrial F0F1ATPsynthase: a structural proteomic analysis. Proteomics 6:921–926

Dickson VK, Silvester JA, Fearnley IM, Leslie AG, Walker JE (2006) On the structure of the stator of the mitochondrial ATP synthase. EMBO J 25:2911–2918

Dreher D, Junod AF (1996) Role of oxygen free radicals in cancer development. Eur J Cancer 32A:30–38

Fang JK, Prabu SK, Sepuri NB, Raza H, Anandatheerthavarada HK, Galati D, Spear J, Avadhani NG (2007) Site specific phosphorylation of cytochrome c oxidase subunits I, IVi1 and Vb in rabbit hearts subjected to ischemia/reperfusion. FEBS Lett 581:1302–1310

Ferguson-Miller S, Brautigan DL, Margoliash E (1976) Correlation of the kinetics of electron transfer activity of various eukaryotic cytochromes c with binding to mitochondrial cytochrome c oxidase. J Biol Chem 251:1104–1115

Fukuda R, Zhang H, Kim JW, Shimoda L, Dang CV, Semenza GL (2007) HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells. Cell 129:111–122

Fukumura D, Kashiwagi S, Jain RK (2006) The role of nitric oxide in tumour progression. Nat Rev Cancer 6:521–534

Gibbons C, Montgomery MG, Leslie AG, Walker JE (2000) The structure of the central stalk in bovine F(1)-ATPase at 2.4 A resolution. Nat Struct Biol 7:1055–1061

Giulivi C (1998) Functional implications of nitric oxide produced by mitochondria in mitochondrial metabolism. Biochem J 332(Pt 3):673–679

Giulivi C (2007) Mitochondria as generators and targets of nitric oxide. Novartis Found Symp 287:92–100 (discussion 100–104)

Green K, Brand MD, Murphy MP (2004) Prevention of mitochondrial oxidative damage as a therapeutic strategy in diabetes. Diabetes 53(Suppl. 1):S110–S118

Guo D, Nguyen T, Ogbi M, Tawfik H, Ma G, Yu Q, Caldwell RW, Johnson JA (2007) Protein kinase C-epsilon coimmunoprecipitates with cytochrome oxidase subunit IV and is associated with improved cytochrome-c oxidase activity and cardioprotection. Am J Physiol Heart Circ Physiol 293:H2219–H2230

Hagen TM, Yowe DL, Bartholomew JC, Wehr CM, Do KL, Park JY, Ames BN (1997) Mitochondrial decay in hepatocytes from old rats: membrane potential declines, heterogeneity and oxidants increase. Proc Natl Acad Sci U S A 94:3064–3069

Helling S, Vogt S, Rhiel A, Ramzan R, Wen L, Marcus K, Kadenbach B (2008) Phosphorylation and kinetics of mammalian cytochrome c oxidase. Mol Cell Proteomics 7:1714–1724

Hennig B (1975) Change of cytochrome c structure during development of the mouse. Eur J Biochem 55:167–183

Hoek JB, Nicholls DG, Williamson JR (1980) Determination of the mitochondrial protonmotive force in isolated hepatocytes. J Biol Chem 255:1458–1464

Hojlund K, Wrzesinski K, Larsen PM, Fey SJ, Roepstorff P, Handberg A, Dela F, Vinten J, McCormack JG, Reynet C, Beck-Nielsen H (2003) Proteome analysis reveals phosphorylation of ATP synthase beta -subunit in human skeletal muscle and proteins with potential roles in type 2 diabetes. J Biol Chem 278:10436–10442

Hoozemans JJ, Veerhuis R, Rozemuller AJ, Eikelenboom P (2002) The pathological cascade of Alzheimer's disease: the role of inflammation and its therapeutic implications. Drugs Today (Barc) 38:429–443

Hopper RK, Carroll S, Aponte AM, Johnson DT, French S, Shen RF, Witzmann FA, Harris RA, Balaban RS (2006) Mitochondrial matrix phosphoproteome: effect of extra mitochondrial calcium. Biochemistry 45:2524–2536

Hüttemann M, Kadenbach B, Grossman LI (2001) Mammalian subunit IV isoforms of cytochrome c oxidase. Gene 267:111–123

Hüttemann M, Jaradat S, Grossman LI (2003) Cytochrome c oxidase of mammals contains a testes-specific isoform of subunit VIb—the counterpart to testes-specific cytochrome c? Mol Reprod Dev 66:8–16

Hüttemann M, Lee I, Liu J, Grossman LI (2007a) Transcription of mammalian cytochrome c oxidase subunit IV-2 is controlled by a novel conserved oxygen responsive element. FEBS J 274:5737–5748

Hüttemann M, Lee I, Samavati L, Yu H, Doan JW (2007b) Regulation of mitochondrial oxidative phosphorylation through cell signaling. Biochim Biophys Acta 1773:1701–1720

Hüttemann M, Lee I, Kreipke CW, Petrov T (2008) Suppression of the inducible form of nitric oxide synthase prior to traumatic brain injury improves cytochrome c oxidase activity and normalizes cellular energy levels. Neuroscience 151:148–154

Iwata S, Lee JW, Okada K, Lee JK, Iwata M, Rasmussen B, Link TA, Ramaswamy S, Jap BK (1998) Complete structure of the 11-subunit bovine mitochondrial cytochrome bc1 complex. Science 281:64–71

Kadenbach B, Arnold S, Lee I, Hüttemann M (2004) The possible role of cytochrome c oxidase in stress-induced apoptosis and degenerative diseases. Biochim Biophys Acta 1655:400–408

Kagan VE, Tyurin VA, Jiang J, Tyurina YY, Ritow VB, Amoscato AA, Osipov AN, Belikova NA, Kapralov AA, Kini V, Vlasova II, Zhao Q, Zou M, Di P, Svistunenko DA, Kurnikov IV, Borisenko GG (2005) Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. Nat Chem Biol 1:223–232

Kaim G, Dimroth P (1999) ATP synthesis by F-type ATP synthase is obligatorily dependent on the transmembrane voltage. EMBO J 18:4118–4127

Kameoka M, Kimura T, Ikuta K (1993) Superoxide enhances the spread of HIV-1 infection by cell-to-cell transmission. FEBS Lett 331:182–186

Ko YH, Pan W, Inoue C, Pedersen PL (2002) Signal transduction to mitochondrial ATP synthase: evidence that PDGF-dependent phosphorylation of the delta-subunit occurs in several cell lines, involves tyrosine, and is modulated by lysophosphatidic acid. Mitochondrion 1:339–348

Korshunov SS, Skulachev VP, Starkov AA (1997) High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. FEBS Lett 416:15–18

Labajova A, Vojtiskova A, Krivakova P, Kofranek J, Drahota Z, Houstek J (2006) Evaluation of mitochondrial membrane potential using a computerized device with a tetraphenylphosphonium-selective electrode. Anal Biochem 353:37–42

Lambert AJ, Brand MD (2004) Superoxide production by NADH: ubiquinone oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner membrane. Biochem J 382:511–517

Lee I, Salomon AR, Ficarro S, Mathes I, Lottspeich F, Grossman LI, Hüttemann M (2005) cAMP-dependent tyrosine phosphorylation of subunit I inhibits cytochrome c oxidase activity. J Biol Chem 280:6094–6100

Lee I, Salomon AR, Yu K, Doan JW, Grossman LI, Hüttemann M (2006) New prospects for an old enzyme: mammalian cytochrome c is tyrosine-phosphorylated in vivo. Biochemistry 45:9121–9128

Lenaz G, Genova ML (2006) Kinetics of integrated electron transfer in the mitochondrial respiratory chain: random collisions versus solid state electron channeling. Am J Physiol Cell Physiol 292: C1221–C1239

Liu SS (1999) Cooperation of a “reactive oxygen cycle” with the Q cycle and the proton cycle in the respiratory chain—superoxide generating and cycling mechanisms in mitochondria. J Bioenerg Biomembr 31:367–376

Liu Z, Lin H, Ye S, Liu QY, Meng Z, Zhang CM, Xia Y, Margoliash E, Rao Z, Liu XJ (2006) Remarkably high activities of testicular cytochrome c in destroying reactive oxygen species and in triggering apoptosis. Proc Natl Acad Sci U S A 103:8965–8970

McIntosh DB, Parrish JC, Wallace CJ (1996) Definition of a nucleotide binding site on cytochrome c by photoaffinity labeling. J Biol Chem 271:18379–18386

Mitchell P (1961) Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. Nature 191:144–148

Miyazaki T, Neff L, Tanaka S, Horne WC, Baron R (2003) Regulation of cytochrome c oxidase activity by c-Src in osteoclasts. J Cell Biol 160:709–718

Miyazaki T, Tanaka S, Sanjay A, Baron R (2006) The role of c-Src kinase in the regulation of osteoclast function. Mod Rheumatol 16:68–74

Moncada S, Bolanos JP (2006) Nitric oxide, cell bioenergetics and neurodegeneration. J Neurochem 97:1676–1689

Moreira PI, Santos MS, Moreno A, Oliveira C (2001) Amyloid beta-peptide promotes permeability transition pore in brain mitochondria. Biosci Rep 21:789–800

Napiwotzki J, Shinzawa-Itoh K, Yoshikawa S, Kadenbach B (1997) ATP and ADP bind to cytochrome c oxidase and regulate its activity. Biol Chem 378:1013–1021

Nicholls DG (1974) 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 50:305–315

Nicholls DG (2006) Simultaneous monitoring of ionophore- and inhibitor-mediated plasma and mitochondrial membrane potential changes in cultured neurons. J Biol Chem 281:14864–14874

Nicholls DG, Ferguson SJ (1992) Bioenergetics 2. Academic Press Limited, London, San Diego

Nobes CD, Brown GC, Olive PN, Brand MD (1990) Non-ohmic proton conductance of the mitochondrial inner membrane in hepatocytes. J Biol Chem 265:12903–12909

Ogbi M, Johnson JA (2006) Protein kinase Cepsilon interacts with cytochrome c oxidase subunit IV and enhances cytochrome c oxidase activity in neonatal cardiac myocyte preconditioning. Biochem J 393:191–199

Ogbi M, Chew CS, Pohl J, Stuchlik O, Ogbi S, Johnson JA (2004) Cytochrome c oxidase subunit IV as a marker of protein kinase Cepsilon function in neonatal cardiac myocytes: implications for cytochrome c oxidase activity. Biochem J 382:923–932

Olinski R, Gackowski D, Foksinski M, Rozalski R, Roszkowski K, Jaruga P (2002) Oxidative DNA damage: assessment of the role in carcinogenesis, atherosclerosis, and acquired immunodeficiency syndrome. Free Radic Biol Med 33:192–200

Ostermeier C, Iwata S, Ludwig B, Michel H (1995) Fv fragment-mediated crystallization of the membrane protein bacterial cytochrome c oxidase. Nat Struct Biol 2:842–846

Ozawa T (1997) Genetic and functional changes in mitochondria associated with aging. Physiol Rev 77:425–464

Pereverzev MO, Vygodina TV, Konstantinov AA, Skulachev VP (2003) Cytochrome c, an ideal antioxidant. Biochem Soc Trans 31:1312–1315

Persichini T, Mazzone V, Polticelli F, Moreno S, Venturini G, Clementi E, Colasanti M (2005) Mitochondrial type I nitric oxide synthase physically interacts with cytochrome c oxidase. Neurosci Lett 384:254–259

Pocsfalvi G, Cuccurullo M, Schlosser G, Scacco S, Papa S, Malorni A (2006) Phosphorylation of B14.5a subunit from bovine heart complex I identified by titanium dioxide selective enrichment and shotgun proteomics. Mol Cell Proteomics 6:231–237

Porteous WK, James AM, Sheard PW, Porteous CM, Packer MA, Hyslop SJ, Melton JV, Pang CY, Wei YH, Murphy MP (1998) Bioenergetic consequences of accumulating the common 4977-bp mitochondrial DNA deletion. Eur J Biochem 257:192–201

Prabu SK, Anandatheerthavarada HK, Raza H, Srinivasan S, Spear JF, Avadhani NG (2006) Protein kinase A-mediated phosphorylation modulates cytochrome c oxidase function and augments hypoxia and myocardial ischemia-related injury. J Biol Chem 281:2061–2070

Rastogi VK, Girvin ME (1999) Structural changes linked to proton translocation by subunit c of the ATP synthase. Nature 402:263–268

Robb-Gaspers LD, Burnett P, Rutter GA, Denton RM, Rizzuto R, Thomas AP (1998) Integrating cytosolic calcium signals into mitochondrial metabolic responses. EMBO J 17:4987–5000

Roberts VA, Pique ME (1999) Definition of the interaction domain for cytochrome c on cytochrome c oxidase. III. Prediction of the docked complex by a complete, systematic search. J Biol Chem 274:38051–38060

Sanishvili R, Volz KW, Westbrook EM, Margoliash E (1995) The low ionic strength crystal structure of horse cytochrome c at 2.1 A resolution and comparison with its high ionic strength counterpart. Structure 3:707–716

Sazanov LA, Hinchliffe P (2006) Structure of the hydrophilic domain of respiratory complex I from *Thermus thermophilus*. Science 311:1430–1436

Schägger H (2002) Respiratory chain supercomplexes of mitochondria and bacteria. Biochim Biophys Acta 1555:154–159

Schägger H, Pfeiffer K (2000) Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. EMBO J 19:1777–1783

Schilling B, Aggeler R, Schulenberg B, Murray J, Row RH, Capaldi RA, Gibson BW (2005) Mass spectrometric identification of a novel phosphorylation site in subunit NDUFA10 of bovine mitochondrial complex I. FEBS Lett 579:2485–2490

Schilling B, Murray J, Yoo CB, Row RH, Cusack MP, Capaldi RA, Gibson BW (2006) Proteomic analysis of succinate dehydrogenase and ubiquinol-cytochrome c reductase (Complex II and III) isolated by immunoprecipitation from bovine and mouse heart mitochondria. Biochim Biophys Acta 1762:213–222

Shears SB, Kirk CJ (1984) Characterization of a rapid cellular-fractionation technique for hepatocytes. Application in the measurement of mitochondrial membrane potential in situ. Biochem J 219:375–382

Steenaart NA, Shore GC (1997) Mitochondrial cytochrome c oxidase subunit IV is phosphorylated by an endogenous kinase. FEBS Lett 415:294–298

Stepp DW (2006) Impact of obesity and insulin resistance on vasomotor tone: nitric oxide and beyond. Clin Exp Pharmacol Physiol 33:407–414

Steverding D, Kadenbach B (1991) 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 266:8097–8101

Suarez MD, Revzin A, Narlock R, Kempner ES, Thompson DA, Ferguson-Miller S (1984) The functional and physical form of mammalian cytochrome c oxidase determined by gel filtration, radiation inactivation, and sedimentation equilibrium analysis. J Biol Chem 259:13791–13799

Sun F, Huo X, Zhai Y, Wang A, Xu J, Su D, Bartlam M, Rao Z (2005) Crystal structure of mitochondrial respiratory membrane protein complex II. Cell 121:1043–1057

Technikova-Dobrova Z, Sardanelli AM, Speranza F, Scacco S, Signorile A, Lorusso V, Papa S (2001) Cyclic adenosine monophosphate-dependent phosphorylation of mammalian mito-

chondrial proteins: enzyme and substrate characterization and functional role. Biochemistry 40:13941–13947

Tsukihara T, Aoyama H, Yamashita E, Tomizaki T, Yamaguchi H, Shinzawa-Itoh K, Nakashima R, Yaono R, Yoshikawa S (1996) The whole structure of the 13-subunit oxidized cytochrome c oxidase at 2.8 A. Science 272:1136–1144

Tsukihara T, Shimokata K, Katayama Y, Shimada H, Muramoto K, Aoyama H, Mochizuki M, Shinzawa-Itoh K, Yamashita E, Yao M, Ishimura Y, Yoshikawa S (2003) The low-spin heme of cytochrome c oxidase as the driving element of the proton-pumping process. Proc Natl Acad Sci U S A 100:15304–15309

Tyurina YY, Kini V, Tyurin VA, Vlasova II, Jiang J, Kapralov AA, Belikova NA, Yalowich JC, Kurnikov IV, Kagan VE (2006) Mechanisms of cardiolipin oxidation by cytochrome c: relevance to pro- and antiapoptotic functions of etoposide. Mol Pharmacol 70:706–717

Vijayasarathy C, Biunno I, Lenka N, Yang M, Basu A, Hall IP, Avadhani NG (1998) Variations in the subunit content and catalytic activity of the cytochrome c oxidase complex from different tissues and different cardiac compartments. Biochim Biophys Acta 1371:71–82

Wan B, Doumen C, Duszynski J, Salama G, Vary TC, LaNoue KF (1993) Effects of cardiac work on electrical potential gradient across mitochondrial membrane in perfused rat hearts. Am J Physiol 265:H453–H460

Wolff SP (1993) Diabetes mellitus and free radicals. Free radicals, transition metals and oxidative stress in the aetiology of diabetes mellitus and complications. Br Med Bull 49:642–652

Yu H, Lee I, Salomon AR, Yu K, Hüttemann M (2008) Mammalian liver cytochrome c is tyrosine-48 phosphorylated in vivo, inhibiting mitochondrial respiration. Biochim Biophys Acta 1777:1066–1071

Zhang H, Huang HM, Carson RC, Mahmood J, Thomas HM, Gibson GE (2001) Assessment of membrane potentials of mitochondrial populations in living cells. Anal Biochem 298:170–180