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## Regulation of cytochrome c oxidase contributes to health and optimal life

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### Abstract

The generation of cellular energy in the form of ATP occurs mainly in mitochondria by oxidative phosphorylation. Cytochrome c oxidase (CytOx), the oxygen accepting and rate-limiting step of the respiratory chain, regulates the supply of variable ATP demands in cells by "allosteric ATP-inhibition of CytOx." This mechanism is based on inhibition of oxygen uptake of CytOx at high ATP/ADP ratios and low ferrocyanochrome c concentrations in the mitochondrial matrix *via* cooperative interaction of the two substrate binding sites in dimeric CytOx. The mechanism keeps mitochondrial membrane potential  $\Delta\Psi_m$  and reactive oxygen species (ROS) formation at low healthy values. Stress signals increase cytosolic calcium leading to  $Ca^{2+}$ -dependent dephosphorylation of CytOx subunit I at the cytosolic side accompanied by switching off the allosteric ATP-inhibition and monomerization of CytOx. This is followed by increase of  $\Delta\Psi_m$  and formation of ROS. A hypothesis is presented suggesting a dynamic change of binding of NDUFA4, originally identified as a subunit of complex I, between monomeric CytOx (active state with high  $\Delta\Psi_m$ , high ROS and low efficiency) and complex I (resting state with low  $\Delta\Psi_m$ , low ROS and high efficiency).

**Key Words:** Cytochrome c oxidase; Regulation of respiration; Allosteric ATP-inhibition; NDUFA4; Reversible phosphorylation; Efficiency of ATP synthesis; Dimerization of cytochrome c oxidase

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**Core Tip:** This article describes the "allosteric ATP-inhibition of cytochrome c oxidase," which prevents the formation of reactive oxygen species (ROS) under resting conditions in all eukaryotic cells by keeping the mitochondrial membrane potential  $\Delta\Psi_m$  at low values. Under stress – *via* increased calcium concentrations – this mechanism is switched off, accompanied by increased rates of ATP-synthesis with decreased efficiency and formation

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of deleterious ROS. A hypothesis is described in which NDUFA4 changes its position from complex I to cytochrome c oxidase when the metabolic state changes from the rest to excited state under stress.

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## INTRODUCTION

All expressions of life require energy in the form of ATP, the universal energy currency of living cells. Large variations in ATP turnover with rates up to 100-fold in skeletal muscle<sup>[1,2]</sup> occur in cells depending on the tissue. ATP is mostly produced in mitochondria by oxidative phosphorylation. In four enzyme complexes of the respiratory chain in the inner membrane of mitochondria, electrons from NADH and FADH<sub>2</sub>, the reduced equivalents of nutrients, are successively transferred via the final complex IV = cytochrome c oxidase (CytOx) to molecular oxygen (O<sub>2</sub>), forming water in a strongly exergonic reaction. In three complexes, complex I (NADH dehydrogenase), complex III (ubiquinol: Cytochrome c oxidoreductase) and complex IV, the energy of this “cold combustion” of nutrients is released in an electrochemical proton gradient  $\Delta\mu\text{H}^+$  across the membrane. Peter Mitchell described the energy of  $\Delta\mu\text{H}^+$  as proton motive force  $\Delta p = \Delta\mu\text{H}^+ / F$  ( $F = \text{Faraday constant}$ ), consisting of an electrical ( $\Delta\Psi_m$ ) and a chemical part ( $\Delta p\text{H}_m$ ):  $\Delta p = \Delta\Psi_m - 59\Delta p\text{H}_m$  (mV)<sup>[3]</sup>. However, most of the proton motive force in mitochondria consists of  $\Delta\Psi_m$  and reaches values of 180-200 mV in isolated mitochondria at state 4 (controlled state of respiration at limited ADP)<sup>[4,5]</sup>. The energy of  $\Delta\mu\text{H}^+$  is used by the ATP synthase (complex V), *via* backward flow of protons, to drive the endergonic reaction: ADP + phosphate  $\rightarrow$  ATP. In the active state 3 of isolated mitochondria (presence of ADP),  $\Delta\Psi_m$  is lower (130-140 mV), since it is partly consumed by the ATP synthase.

## REACTIVE OXYGEN SPECIES IN MITOCHONDRIA

Health and optimal life are frequently hurt by the consequences of psychosocial stress. The consequences appear in cells as “oxidative stress” caused by the over-production of reactive oxygen species (ROS, mainly O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub>) in mitochondria. While low amounts of ROS have in cells signaling functions<sup>[6,7]</sup>, high amounts produced in mitochondria are generally assumed to participate in aging<sup>[8-10]</sup> and in the generation of numerous diseases including cancer, hypertension, atherosclerosis, ischemia / reperfusion injury, neurodegenerative diseases like Alzheimer's and Parkinson's disease, rheumatoid arthritis, diabetes mellitus, and mitochondrial diseases<sup>[11-14]</sup>.

It was found that ROS are generated in the respiratory chain at increasing  $\Delta\Psi_m$  - values above 130-140 mV<sup>[15-17]</sup>. The superoxide radical anion O<sub>2</sub><sup>-</sup> is mostly produced at complexes I and III, due to the transfer of a single electron to O<sub>2</sub><sup>[18,19]</sup>, and is immediately converted into H<sub>2</sub>O<sub>2</sub> by mitochondrial superoxide dismutases<sup>[20]</sup>.

In most situations of animals, *e.g.*, during sleep, the resting state predominates with high ATP-levels, low amounts of ADP, and low consumption of ATP (at least in skeletal muscles). According to the results with isolated mitochondria at these low rates of ATP consumption (state 4, with  $\Delta\Psi_m$  values of 180-200 mV<sup>[4,5]</sup>), large amounts of ROS would be produced under resting conditions. Fortunately in resting living cells, mitochondrial  $\Delta\Psi_m$  values are low, between 100 and 130 mV (for references see<sup>[21]</sup>). These low  $\Delta\Psi_m$  values are sufficient for maximal rates of ATP synthesis, since the rate of ATP synthesis by the ATP synthase is saturated and maximal at 100-120 mV<sup>[22]</sup>. But how are these low  $\Delta\Psi_m$  values of 100-130 mV achieved to maintain a healthy life?

It was found, however, that under various stress conditions a transient increase of the mitochondrial membrane potential does occur, called “hyperpolarization,” which in some cases is followed by cell apoptosis<sup>[23]</sup>. Both, the low  $\Delta\Psi_m$  values of 100-130 mV in resting living cells and the hyperpolarization of  $\Delta\Psi_m$  under stress are explained below by the “allosteric ATP-inhibition of CytOx.”

## CYTOCHROME C OXIDASE, A CONTROLLING POINT OF OXIDATIVE METABOLISM

CytOx developed early during evolution as the final oxygen accepting enzyme of respiratory chains for the generation of ATP by oxidative phosphorylation<sup>[24]</sup>. With increasing organismal complexity during evolution the number of protein subunits in the CytOx complex increased from 2-3 in bacteria over 7 in the slime mold *Dictiostelium discoideum*, and 11 in yeast to 13 in mammals<sup>[25]</sup>. In eukaryotes the “catalytic” subunits I-III are encoded on mitochondrial DNA and synthesized in mitochondria. The additional “supernumerary” subunits are encoded by the nuclear DNA and synthesized on cytoplasmic ribosomes. A complicated machinery is required for the transport of these subunits into mitochondria<sup>[26]</sup> and for the assembly into the 13-subunit CytOx complex of vertebrates<sup>[27]</sup>.

In contrast to many other “oxidases”<sup>[28]</sup>, CytOx produces no ROS during reduction of dioxygen, due to its unique binding site for O<sub>2</sub> in subunit I, composed of heme a<sub>3</sub>, Cu<sub>B</sub> and a tyrosyl-group, allowing simultaneous transfer of 4 electrons to O<sub>2</sub><sup>[29]</sup>. The binding site for cytochrome *c* containing two copper atoms is located in subunit II, and subunit three stabilizes the core subunits. The catalytic center of CytOx, located in subunits I-III, is very similar in bacteria and in eukaryotes, and the basic functions, *i.e.* reduction of oxygen<sup>[30]</sup> and generation of an electrochemical potential  $\Delta\mu\text{H}^+$ <sup>[31]</sup> are the same. Therefore the role of “supernumerary” subunits in the activity of CytOx was ignored. In the fourth edition of their textbook “Bioenergetics4”<sup>[32]</sup>, Nicholls and Ferguson denied the catalytic function of the supernumerary subunits. However, by the use of subunit-specific antibodies for 3 of the ten nuclear encoded subunits a specific function on the activity of CytOx was demonstrated. In subunit IV: The “allosteric ATP-inhibition” *via* binding of ATP at its matrix domain at high ATP/ADP-ratios<sup>[33]</sup>, and also in subunit IV: The decrease of cytochrome *c* affinity by binding ATP to the intermembrane domain at high ATP/ADP-ratios<sup>[34]</sup>. In subunit Va: The abolishment of the “allosteric ATP-inhibition” by binding of 3,5-diiodothyronine<sup>[35]</sup>, and in subunit VIa-heart isoform: The decrease of H<sup>+</sup>/e<sup>-</sup>-stoichiometry from 1 to 0.5 at high ATP/ADP-ratios<sup>[36]</sup>.

From application of the metabolic control analysis to isolated mitochondria<sup>[37-39]</sup> a 5- to 7-fold excess of CytOx capacity was found over the amount required to support the endogenous respiration of mitochondria<sup>[40-42]</sup>. However, later studies with intact cells demonstrated that CytOx represents the rate limiting step of oxidative phosphorylation in living cells<sup>[43,44]</sup>.

## FEEDBACK INHIBITION OF CYTOX BY ATP: THE “ALLOSTERIC ATP-INHIBITION OF CYTOX“

The “allosteric ATP-inhibition of CytOx“ based on the exchange of bound ADP by ATP at the matrix domain of CytOx subunit IV-1 at high ATP/ADP ratios originally discovered in 1997<sup>[32,45]</sup>, represents a feedback inhibition of mitochondrial respiration by its final product ATP. We have described this mechanism in more than 20 publications and discussed its implications on human health more recently<sup>[46-48]</sup>.

The bovine heart enzyme contains 10 high-affinity binding sites for ADP seven of which are exchanged by ATP at high ATP/ADP ratios<sup>[34]</sup>. The exchange of bound ADP by ATP at high ATP/ADP-ratios (half-maximal at ATP/ADP = 28) induces a sigmoidal inhibition curve in the kinetics of oxygen uptake *vs* ferrocytochrome *c* concentration (Hill-coefficient = 2<sup>[45]</sup>). This kinetic behaviour indicates a cooperativity between two binding sites of the substrate ferrocytochrome *c*. Since the CytOx monomer contains only one binding site for cytochrome C<sup>[49]</sup>, a dimeric CytOx structure is required for the feedback inhibition of CytOx activity by the “allosteric effector” ATP. At lower ATP/ADP-ratios the CytOx kinetics exhibits normal hyperbolic saturation curves. The allosteric ATP-inhibition is independent of  $\Delta\Psi_m$ <sup>[45]</sup>. The ATP/ADP ratio in the mitochondrial matrix for half-maximal inhibition of CytOx activity at ATP/ADP = 28<sup>[45]</sup> corresponds to the high cytosolic ATP/ADP ratio of 100-1000 determined by <sup>31</sup>P-NMR measurements in rat heart<sup>[50]</sup>. Due to  $\Delta\Psi_m$  the ATP/ADP-ratio in the mitochondrial matrix will be lower (ATP/ADP = 4-40, see<sup>[47]</sup>).

The first crystal structure of CytOx was a dimer<sup>[51]</sup>. But the structure of the physiological relevant CytOx dimer must be slightly different because in the crystals 10 molecules of cholate are bound per CytOx monomer<sup>[52]</sup>. The exchange of cholate by ADP in the cholate-CytOx is a slow process and accompanied by a spectral change<sup>[52]</sup>.

This contrasts with the immediate exchange of bound ATP by ADP in the ADP-CytOx<sup>[53]</sup> which indicates the non-physiological structure of the cholate-CytOx crystals<sup>[51]</sup>. In fact, the crystallisation of the native ADP-CytOx or ATP-CytOx appears not possible. Only by using cholate enough CytOx could be obtained for crystallization (Kyoko Shinzawa-Itoh, personal communication). The control of respiration by the allosteric ATP-inhibition of CytOx, also named “second mechanism of respiratory control”<sup>[54]</sup>, is independent of  $\Delta\Psi_m$ <sup>[45]</sup>, in contrast to the classical “respiratory control” where mitochondrial respiration is limited at high  $\Delta\Psi_m$  values<sup>[5]</sup>.

The allosteric ATP-inhibition of CytOx keeps  $\Delta\Psi_m$  at low values (< 130 mV), due to feedback inhibition of CytOx activity by ATP at high ATP/ADP-ratios, preventing further increase of  $\Delta\Psi_m$  by proton pumping within complexes I, III, and IV of the respiratory chain. The inhibitory effect of ATP on  $\Delta\Psi_m$  has also been measured directly in isolated rat liver mitochondria using a tetraphenyl phosphonium electrode<sup>[55]</sup>. The low ROS production in mitochondria of living cells under resting conditions<sup>[18]</sup> is thus explained by the allosteric ATP-inhibition of CytOx which maintains low  $\Delta\Psi_m$  values<sup>[46]</sup>. Therefore, this mechanism contributes to the health and optimal life of higher organisms.

It was suggested that the allosteric ATP-inhibition of CytOx contributes to an optimal efficiency of oxidative phosphorylation and is switched off under stress and excessive work in order to increase the rate of ATP synthesis which is accompanied by lower efficiency<sup>[47]</sup>. Furthermore, it was assumed that higher efficiency may be achieved by increased H<sup>+</sup>/e<sup>-</sup>-stoichiometry of proton pumping in CytOx<sup>[46]</sup>. In general, a constant H<sup>+</sup>/e<sup>-</sup> = 1 was assumed for CytOx<sup>[56,57]</sup>. The Yoshikawa group identified in bovine heart CytOx a third proton channel, the H-channel<sup>[58-61]</sup> which is absent in bacterial CytOx<sup>[62]</sup>. We suggested that the allosteric ATP-inhibition which maintains low  $\Delta\Psi_m$  values could increase the H<sup>+</sup>/e<sup>-</sup>-stoichiometry of CytOx to 2, based on additional proton pumping through the H-channel which is energetically possible<sup>[47]</sup>. In fact, a H<sup>+</sup>/e<sup>-</sup>-stoichiometry of 2 was previously measured for CytOx in isolated rat liver mitochondria<sup>[63-66]</sup>.

In bovine heart mitochondria, most CytOx (> 85%) occurs as free complexes<sup>[67]</sup> not assembled into supercomplexes like respirasomes<sup>[68]</sup>. In the respirasome I<sub>1</sub>III<sub>2</sub>IV<sub>1</sub> CytOx appears as monomer<sup>[69,70]</sup> where the binding site between the two monomers in the dimeric crystal structure<sup>[51,71]</sup> is free and allows dimerization of two respirasomes. This holds also for the megacomplex I<sub>2</sub>III<sub>2</sub>IV<sub>2</sub><sup>[72]</sup>.

The allosteric ATP-inhibition of CytOx is active in most cell types which express subunit IV-1. The isoform subunit IV-2 was found to be expressed in human cell lines under hypoxia<sup>[73]</sup>. Also in isolated astrocytes and cerebellar granule cells subunit IV-2 is expressed under hypoxic conditions accompanied by an abolition of the allosteric inhibition of CytOx by ATP<sup>[74]</sup>.

## STRESS TURNS OFF THE ALLOSTERIC ATP-INHIBITION OF CYTOX VIA CYTOSOLIC CALCIUM

The fact that the feedback inhibition of CytOx by ATP has been ignored for more than 15 years is also based on its unique biochemical properties. It was found to be dependent on phosphorylation of CytOx subunit I at the cytosolic side. After dephosphorylation of this site by a calcium-activated protein phosphatase (PP1) the allosteric ATP-inhibition of CytOx is switched off. Rephosphorylation by a cAMP-dependent protein kinase (PKA) switches it on again. These observations were made with the isolated enzyme which was partly reconstituted in liposomes<sup>[75-77]</sup>. Recently these properties could also be shown with intact rat heart mitochondria. In this study a very low concentration of calcium (1-10 micromolar) was sufficient to switch off the allosteric ATP-inhibition<sup>[1]</sup>. Various stress signals, including psychosocial stress, increase the cytosolic calcium concentration and activate a Ca<sup>2+</sup>-dependent protein phosphatase which is located in the mitochondrial intermembrane space leading to dephosphorylation of CytOx at the cytosolic side of subunit I<sup>[76]</sup>. This dephosphorylation is accompanied by loss of the allosteric ATP-inhibition, an increase of  $\Delta\Psi_m$  and ROS formation<sup>[48]</sup>.

In conclusion, the “allosteric ATP-inhibition of CytOx” has four physiological functions: (1) To maintain a constant high ATP/ADP ratio in cells; (2) To inhibit the oxygen consumption of mitochondria when sufficient ATP is available; (3) To prevent the formation of ROS under resting conditions by keeping the mitochondrial membrane potential  $\Delta\Psi_m$  at low values; and (4) To increase the rate of respiration and ATP synthesis during excessive workload or stress by switching it off. This is

accompanied by reduced efficiency and generation of deleterious ROS.

## NDUFA4, A RESPIRATORY CHAIN-ASSOCIATED FACTOR

NDUFA4 was identified as a nuclear-encoded subunit of complex I<sup>[78-80]</sup>. However, together with two other subunits its gene had significantly increased amino acid substitution rates during primate radiation, suggesting that they have been subjected to adaptive selection<sup>[81]</sup>. Later, NDUFA4 was no longer considered a subunit of complex I<sup>[82]</sup>. Recently NDUFA4 was claimed to represent the 14<sup>th</sup> subunit of mammalian CytOx<sup>[83-86]</sup>. The cryo-EM structure of a NDUFA4-CytOx complex could be determined, where NDUFA4 is bound to the CytOx monomer exactly at the binding site between the two monomers in the dimeric enzyme<sup>[87]</sup>. If NDUFA4 would represent an essential subunit of CytOx, a dimeric structure, as determined in CytOx crystals by Tsukihara *et al*<sup>[51]</sup>, would be impossible. We doubted the claim that NDUFA4 represents the 14<sup>th</sup> subunit of mammalian CytOx. This doubt is based on immunoprecipitation of the 13-subunit CytOx from Triton X-100 dissolved rat liver mitochondria<sup>[88]</sup>. In addition, the feedback inhibition of CytOx by ATP *via* cooperativity of two binding sites for cytochrome *c* in the dimeric enzyme (the allosteric ATP-inhibition of CytOx), would be impossible with the monomeric NDUFA4-CytOx complex. Recently we concluded from studies with intact isolated rat heart mitochondria<sup>[1]</sup> that cAMP-dependent phosphorylation at the intermembrane side of CytOx subunit I<sup>[76]</sup> induces a dimeric enzyme with allosteric ATP-inhibition, while calcium-activated dephosphorylation monomerizes CytOx accompanied by abolishment of the allosteric ATP-inhibition and binding of NDUFA4<sup>[87]</sup>. These results strongly suggest that stress-dependent increase of cytosolic calcium leads to a rise of  $\Delta\Psi_m$  and ROS formation at lower efficiency due to loss of the allosteric ATP-inhibition of CytOx<sup>[47]</sup>.

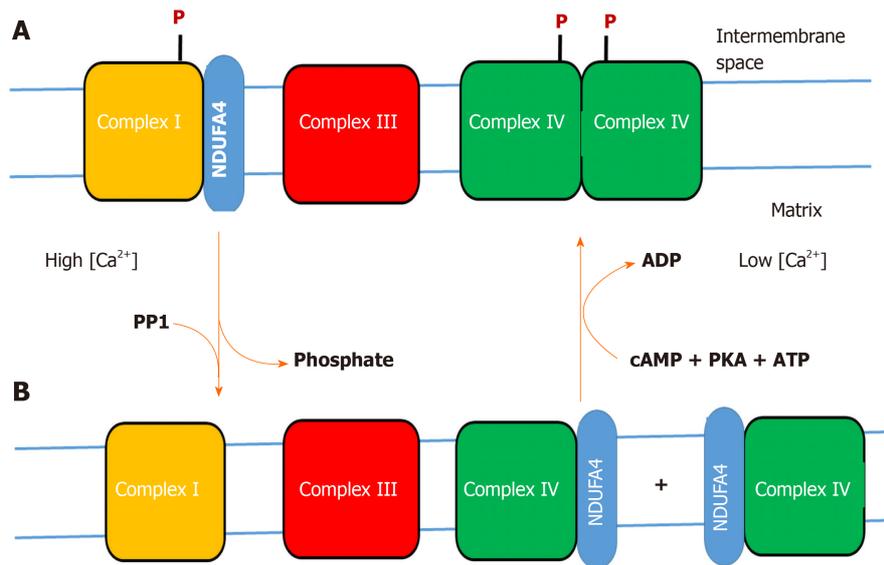
## HYPOTHESIS

The following hypothesis describes a dynamic change of reversible protein-protein interactions which are not expected from X-ray crystal structures or cryo-EM structures but occur frequently in cells (see *e.g.*<sup>[89]</sup>).

We postulate that NDUFA4 (N-terminal amino acid sequence: MLRQ) changes its binding position between complex I and CytOx, depending on the stress situation and/or energetic (ATP) requirements of cells (Figure 1). We postulate that increased cytosolic calcium concentration (> 1 micromolar), as a consequence of various stress factors, activates a calcium-dependent protein phosphatase in the intermembrane space of mitochondria, which dephosphorylates the CytOx dimer, accompanied by monomerization of CytOx, loss of the allosteric ATP-inhibition, and binding of NDUFA4. Also complex I is postulated to be dephosphorylated by a calcium-dependent protein phosphatase, accompanied by decreased affinity of complex I to NDUFA4 which binds to monomeric COX forming the NDUFA4-CytOx complex<sup>[87]</sup>. The binding of NDUFA4 to phosphorylated complex I and its dissociation after dephosphorylation could be tested by BN-PAGE with mitochondria either treated with cAMP or with calcium (see<sup>[1]</sup>), followed by immunodetection with specific antibodies. The physiological function of binding NDUFA4 to (phosphorylated) complex I under relaxed conditions could be to decrease the affinity of complex I to NADH because high NADH/NAD<sup>+</sup>-ratios were shown to stimulate ROS production in complex I<sup>[90-93]</sup>.

Various stress signals were shown to increase cytosolic calcium concentrations in cells including high glutamate<sup>[94]</sup> or glucose<sup>[95]</sup>. In addition, psychosocial stress was shown to increase cytosolic calcium, as shown in cardiomyocytes<sup>[96]</sup>, platelets<sup>[97]</sup>, hippocampal-derived HT22 cells<sup>[98]</sup>, urothelial cells<sup>[99]</sup>, and cardiomyocytes<sup>[100]</sup>. Under resting conditions the cytosolic calcium concentration is low (about 0.1 micromolar), and a cAMP-dependent PKA rephosphorylates CytOx, accompanied by dimerization and switching on the allosteric ATP-inhibition of CytOx. This was shown in previous *in vitro* studies with the isolated CytOx by phosphorylation with cAMP-dependent PKA+ATP. In addition it was switched off by dephosphorylation with a calcium-activated PP1<sup>[76]</sup>. Dimerization of CytOx is possible between single CytOx complexes as well as between respirasomes since the binding domain between monomers in dimeric CytOx<sup>[71]</sup> is free in supercomplexes<sup>[70,72]</sup>.

The function of binding NDUFA4 to monomeric CytOx is still unclear. The change



**Figure 1 Hypothesis on the variable binding of NDUFA4 to complex I or cytochrome c oxidase.** A: Phosphorylation (P) of complex I and cytochrome c oxidase (CytOx) at low cytosolic calcium ( $< 1$  micromolar) stabilizes binding of NDUFA4 to complex I and the function of the “allosteric ATP-inhibition” of dimeric CytOx (resting state); B: Stress-induced increase of cytosolic calcium ( $> 1$  micromolar) dephosphorylates complex I and CytOx by a calcium-activated PP1, accompanied by monomerization of CytOx and switching off its allosteric ATP-inhibition. NDUFA4 is detached from complex I and binds to monomeric CytOx (excited state). At low cytosolic calcium, a cAMP-dependent protein kinase A phosphorylates complex I and CytOx. This changes the binding position of NDUFA4 from CytOx to complex I, accompanied by dimerization of CytOx and activation of its allosteric ATP-inhibition.

of dimeric CytOx to monomeric CytOx (NDUFA4-CytOx) is associated with the loss of allosteric ATP-inhibition and increase of the rate of respiration and ATP synthesis at lower efficiency. The dimeric enzyme composed of two phosphorylated 13-subunit monomers represents CytOx in the resting state with higher efficiency (probably with increased  $\text{H}^+/\text{e}^-$ -stoichiometry = 2)<sup>[47]</sup>. Our view is different from that of Shinzawa-Itoh *et al*<sup>[101]</sup> who described the dimeric CytOx as the physiological standby form in the mitochondrial membrane.

The role of NDUFA4 as 14<sup>th</sup> subunit of CytOx was suggested by<sup>[84]</sup> based on mutations in the *NDUFA4* gene accompanied by defective CytOx activity in patients with Leigh syndrome. In muscle tissue from patients the NDUFA4 protein was absent while the CytOx complex was still there but without activity. Since the literature is full of papers measuring CytOx activity with the isolated 13-subunit enzyme (without NDUFA4), the physiological function of NDUFA4 remains unknown. We suggest to rename it to “mitochondrial respiratory chain associated factor 1”.

## CONCLUSION

A hypothesis is presented suggesting a dynamic change of binding of NDUFA4, originally identified as a subunit of complex I, between monomeric CytOx (active state with high  $\Delta\Psi_{\text{m}}$ , high ROS and low efficiency) and complex I (resting state with low  $\Delta\Psi_{\text{m}}$ , low ROS and high efficiency).

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