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Regulation of mitochondrial energy generation in health and disease

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Abstract

In mammalian cytochrome *c* oxidase (COX) three of the ten nuclear coded subunits (VIa, VIIa, VIII) occur in tissue-specific isoforms. The isoform distribution, however, varies in liver and heart of different species. Subunit VIII is different in liver and heart of bovine, dog, rat and chicken, but identical in human (liver-type) on one hand, and sheep, rabbit and rainbow trout (heart-type) on the other hand, as determined by N-terminal sequencing. Two moles of trinitrophenyl-ATP bind to monomeric COX from bovine heart and one to COX from bovine liver with dissociation equilibrium constant (K_d) values of about 3 μM. One binding site at the heart enzyme is blocked by a monoclonal antibody to subunit VIa-H. ATP (and/or ADP) interact with COX at two or three high-affinity binding sites, as shown by titration of the spectral changes of COX. Isolated COX from bovine heart was reconstituted with variable intraliposomal ATP/ADP ratios. By measuring the RCR (respiratory control ratio) and RCR_{Val} (related to the valinomycin-respiration), which is a direct measure of the H^+/e^--stoichiometry (Wilson and Prochaska, Arch. Biochem. Biophys. 282 (1990) 413–420), almost complete inhibition of the proton pump activity of COX by high intraliposomal ATP concentrations was found. The vectorial uptake of protons for the formation of water, however, appears to be unaffected by nucleotides. This regulatory mechanism is assumed to have physiological significance for thermogenesis in muscle at rest. COX of fibroblasts from patients suffering from Leigh's syndrome, which is associated with a decreased COX activity, are suggested to have an imcompletely assembled enzyme complex. This suggestion is further corroborated by the higher temperature-sensitivity of the enzyme when compared with COX from normal control fibroblasts. Defective regulation of COX via nuclear coded subunits is also proposed to cause mitochondrial diseases.

Keywords: Cytochrome *c* oxidase; Nucleotide binding; Tissue-specific isozyme; Subunit composition; Proton pump; Leigh's syndrome

1. Control of mitochondrial ATP synthesis

The synthesis of ATP in mitochondria by oxidative phosphorylation involves four energy transducing enzyme complexes, all representing membrane-bound proton pumps. The respiratory chain contains complex I (NADH-UQ-oxidoreductase), complex III (UQH_2-cytochrome *c*-oxidoreductase) and complex IV (cytochrome *c* oxidase (COX)), which translocate protons from the matrix into the cytosol, while the ATP-synthase (complex V) uses the electrochemical proton gradient across the inner mitochondrial membrane to synthesize ATP. Electron transport from NADH to oxygen along the respiratory chain is accompanied by the free energy change ($\Delta G^{\circ\prime}$) of 69.5, 40.5 and 110 kJ/mol, respectively. The free energy change at complexes I and III together is thus the same as that of complex IV, which represents an almost irreversible step. As pointed out in textbooks, essentially irreversible reactions are potential control sites. The activity of COX is thus expected to be controlled by allosteric effectors, in addition to the control by the concentrations of the substrates ferrocytochrome *c* and oxygen, and the membrane potential.

2. Structure of cytochrome c oxidase (COX)

COX from mammalia is composed of three mitochondrial coded and ten nuclear coded subunits, which partly occur in tissue-specific isoforms [1,2]. The tissue-specific distribution of subunit isoforms, however, varies in different species. Table 1 shows the distribution of the heart- (H) and liver-type (L) of subunits VIa, VIIa and VIII in liver and heart of various species. In liver usually the

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Table 1
Distribution of isoforms of COX subunits VIa, VIIa and VIII in different species

Tissue	Subunit VIa		Subunit VIIa		Subunit VIII	
	Liver	Heart	Liver	Heart	Liver	Heart
Human	L	L+H	L	L+H	L	L
Bovine	L	H	L	H	L	H
Sheep	L	H	L+H	H	H	H
Dog	L	H	L	H	L	H
Rabbit	L	L+H	L	L+H	H	H
Rat	L	L+H	L	L	L	H
Chicken	unknown		L+H	H	L	H
Rainbow trout	absent		L	H	H	H

The data are partly taken from Refs. [3] and [4] and from Freund (R. Freund (1994) Dissertation, Fachbereich Chemie der Philipps-Universität, D-35032 Marburg, Germany). L, liver-type isoform; and H, heart-type isoform.

liver-type of subunits VIa and VIIa occurs. In the heart of some species both, the liver- and heart-type isoforms of subunits VIa and/or VIIa occur together. In human heart, and in particular in human skeletal muscle, the heart-type of subunits VIa and VIIa predominate, but an increase of the heart-type/liver-type ratio was shown during the transition from fetal to adult stage [5]. Of particular interest is the observation, that subunit VIII occurs in liver and heart of some species only in one isoform, which could be either the liver-type (human) or the heart-type (sheep, rabbit and rainbow trout [4]). This result was verified by N-terminal sequencing of the corresponding gel bands, except for dog and chicken, where the apparent molecular weights of stained bands on SDS-PAGE gels were taken to deduce the L- and H-type of subunit VIII. The N-terminal amino acid sequences of subunits VIII from liver and heart of various species are presented in Table 2. The sequences clearly identify the liver- or heart-type of subunit VIII.

Recently we isolated COX from liver and heart of rainbow trout and identified a new pair of tissue-specific isoforms for subunit Vb [4]. The fish enzymes are apparently lacking subunits VIa and VIIb. The lack of subunit

Table 2
N-terminal amino acid sequences of subunits VIII from liver and heart of various species

	Liver	Heart
Human	I H S L P P E G K L [6]	dto [7]
Bovine	I H S K P P R E Q L [8]	I T A K P A R T P T [8]
Rat	V H S K P P R E Q L [9]	I S S K P A K S P T [9]
Sheep	dto [N]	I T A K P A K T P T [N]
Rabbit	dto [N]	I S G K P A R T P T [N]

COX of liver and heart of sheep and rabbit was isolated by standard procedures [10], separated by SDS-PAGE [11], electroblotted onto ProBlott polyvinylidene difluoride membranes (Applied Biosystems), and the bands corresponding to subunit VIII were excised and applied to a model 477A protein sequencer (Applied Biosystems). Numbers in brackets are references, [N] indicates new sequences. dto indicates the same sequence as in the other tissue.

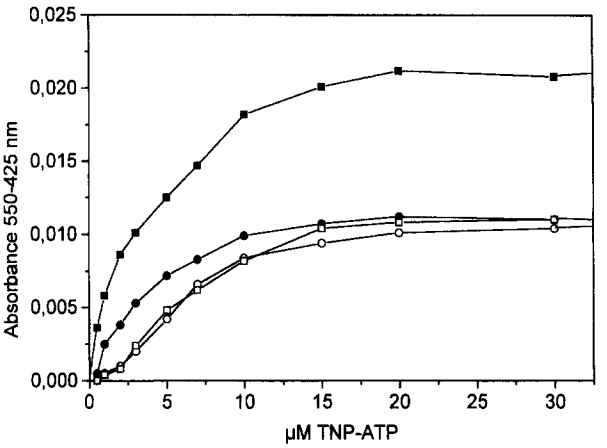

Fig. 1. Spectral changes of TNP-ATP after titration of isolated COX from bovine heart. COX (2 μM), isolated by standard procedures [10], was titrated in tandem cuvettes with TNP-ATP in 10 mM K-Hepes (pH 7.4), 0.05% dodecyl maltoside, as described previously [15]. The TNP-ATP difference spectra were recorded from 350–550 nm 3 min after each addition. Squares, COX from heart; circles, COX from liver. Closed symbols, direct titration of the enzymes with TNP-ATP; open symbols, titration after preincubation with the monoclonal antibody to subunits VIc + VIa-H.

VIa in the exothermic fish is of particular interest, because subunit VIa-H, expressed in heart and skeletal muscle of endothermic mammalia, is shown below to bind specifically nucleotides, and is suggested to participate in muscle-specific thermogenesis at rest. Interestingly subunit VIa occurs also in yeast and has been suggested to interact with ATP [12].

3. Interaction of nucleotides with cytochrome c oxidase

In previous studies on the effects of intraliposomal ADP on the kinetics of reconstituted COX, we have applied a monoclonal antibody which cross-reacts with bovine COX subunits VIc and VIa-H (heart-type) [13]. The cross-reactivity was shown to be due to sequence homologies between these subunits [14]. With COX from bovine liver the antibody reacts only with subunit VIc, not with subunit VIa-L (liver-type) [13]. This antibody was now used to measure the binding of TNP-ATP (2′(or 3′)-*O*-(2,4,6-trinitrophenyl)-ATP) to COX from bovine heart and liver, by following the change of absorbance of TNP-ATP at 425 nm. In a previous study two binding sites for TNP-ATP were identified at COX from bovine heart [15]. As shown in Fig. 1, the binding of TNP-ATP to COX from bovine heart and liver shows saturation at about 20 μM, but the amount of bound TNP-ATP is about twice in heart as compared to liver COX. Preincubation of the enzymes with the monoclonal antibody reduced the binding of TNP-ATP to the heart enzyme by about 50%, down to the level of the liver enzyme, but did not change the binding to the liver enzyme. These data suggest the presence of one

binding site for TNP-ATP at COX from bovine liver and two binding sites at COX from bovine heart, from which one binding site is located at subunit VIa-H.

In further studies the binding of ATP and/or ADP to COX from bovine heart and liver was investigated by measuring the spectral change of the gamma-band of COX. In Fig. 2 the changes of the difference spectra at increasing concentrations of nucleotides are shown, which have a maximum at 425 nm. With both enzymes saturation of the absorbance changes are observed at about 100 μM nucleotide. The titration curves show high- and low-affinity phases. ADP or ATP, added after preincubation with 50 μM nucleotide, results in an additional spectral change with the heart but not with the liver enzyme, indicating different nucleotide binding sites, with preference for either one of the nucleotides, at the heart enzyme. Preliminary data from Scatchard plots on the binding of [^{35}S]ATP to the heart enzyme indicate the presence of one high affinity binding site (K_d about 1 μM) and two low-affinity binding sites (K_d about 20 μM) for ATP at the heart enzyme.

4. Effect of nucleotides on proton pumping

By using the monoclonal antibody to subunits VIc + VIa-H intraliposomal ADP was shown to stimulate the uncoupled respiration of reconstituted COX from bovine heart, but not from bovine liver, via binding to subunit VIa-H [13], and to stimulate the respiratory control ratio of the reconstituted heart enzyme [16]. Wilson and Prochaska [17] described a new parameter, RCR_{Val} (respiratory control ratio: valinomycin + CCCP-respiration/valinomycin-respiration), which is directly related to the H^+/e^--stoichiometry of reconstituted COX — in contrast to the usually measured RCR (valinomycin + CCCP-respiration/control-respiration). We have measured the oxygen uptake of reconstituted COX from bovine heart with and without valinomycin and valinomycin + CCCP at increasing concentrations of cytochrome *c* (Fig. 3). The respiration without uncoupler is saturated at low concentrations of cytochrome *c*, whereas the uncoupled respiration increases with increasing concentrations of cytochrome *c* up to 50 μM. The valinomycin-respiration shows a maximum at about 7 μM cytochrome *c*.

In a second series of experiments we have reconstituted COX from bovine heart in the presence of 10 mM total nucleotides but at varying ATP/ADP ratios, resulting in proteoliposomes with defined intraliposomal (matrix) ATP/ADP ratios. As shown in Fig. 4, the presence of 5% or higher portions of ADP within the vesicles leads to stimulation of the uncoupled respiration by about 40% at 5 μM cytochrome *c* and by about 15% at 50 μM cytochrome *c*. The RCR values of the proteoliposomes are

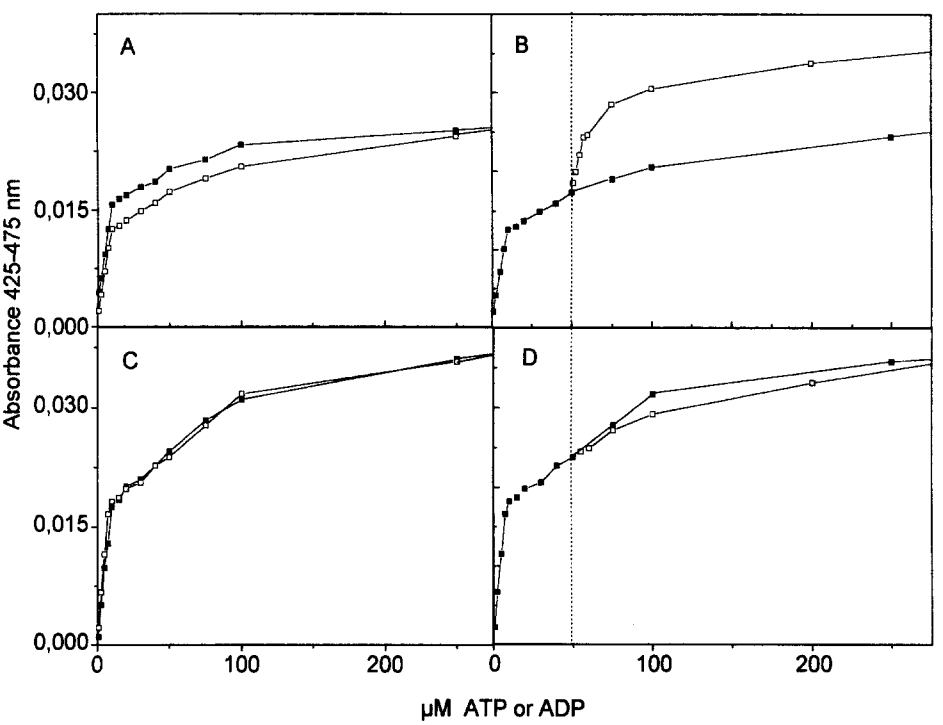

Fig. 2. Spectral changes of COX from bovine heart and liver after titration with ATP and/or ADP. Isolated COX from bovine heart and liver [10] was titrated in tandem cuvettes with the indicated nucleotide in 10 mM K-Hepes (pH 7.4), 0.05% dodecyl maltoside. Difference spectra of COX were recorded from 400–500 nm, 3 min after each addition. (A,B) COX from heart, and (C,D) COX from liver. Closed symbols, titration with ATP; open symbols, titration with ADP. In (B) and (D), in addition to the ATP titration, a second titration is shown, where the titration up to 50 μM ATP was followed by titration with ADP.

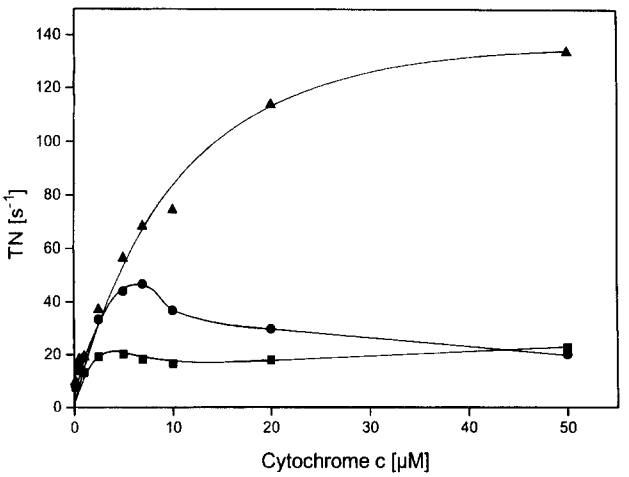

Fig. 3. Respiratory rate of reconstituted COX at increasing concentrations of cytochrome *c* in the absence and presence of valinomycin or valinomycin+CCCP. COX was isolated from bovine heart mitochondria as described [10]. In order to improve the respiratory control ratio the final enzyme was dialyzed against 50 mM K-P_i (pH 7.2), 0.1% Triton X-100 and the DEAE-Sephacel chromatography and ammonium sulfate fractionation were repeated. Reconstitution of COX was performed by the hydrophobic adsorption method [13], in 10 mM K-Hepes (pH 7.4), 40 mM KCl, 1.5% sodium cholate. Respiration of proteoliposome preparations was measured by the polarographic method [13] in 10 mM K-Hepes (pH 7.4), 40 mM KCl, 25 mM Tris-ascorbate, 0.1 to 50 μM cytochrome *c* (Sigma, type VI from horse heart), 0.02 μM reconstituted COX, (i) without additions (squares), (ii) after addition of 1 μg/ml valinomycin (circles), and (iii) after addition of 3 μM CCCP (carbonylcyanide-*m*-chlorophenylhydrazone) (triangles). The rate of respiration is presented as turnover number (TN = mol electrons ($\frac{1}{4}$ O_2) (mol heme aa_3)$^{-1}$ s^{-1}).

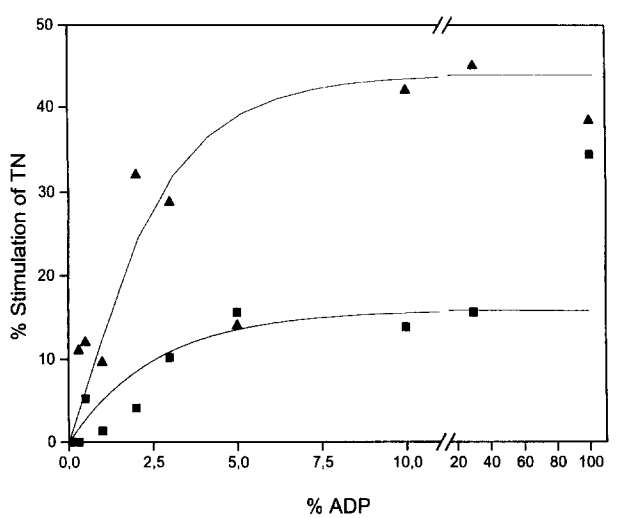

Fig. 4. Increase of uncoupled respiration of reconstituted COX with decreasing intraliposomal ATP and increasing ADP concentrations. Reconstitution of COX was done as described in the legend to Fig. 3, in the presence of various concentrations of ATP and/or ADP, whereby the total (intraliposomal) nucleotide concentration was held constant at 10 mM. The turnover was measured at 5 (triangles) and 50 μM cytochrome *c* (squares) in the presence of 1 μg/ml valinomycin and 3 μM CCCP with proteoliposomes containing the indicated percentage of intraliposomal ADP.

diminished, if the ATP concentration in the vesicles exceeds 95% of total nucleotides (Fig. 5). The RCR decreases from 16 to about 8 at 50 μM cytochrome *c* and from 8 to 3 at 5 μM cytochrome *c*, if the vesicles contain only ATP. The RCR_{Val} is even more affected by intraliposomal nucleotides, which decreases from 8 to 3 at 50 μM cytochrome *c* and from 3 to 1 at 5 μM cytochrome *c* concentrations at 100% (10 mM) intraliposomal ATP. These results suggest that the proton pump activity of COX, represented by RCR_{Val}, is turned off by intraliposomal (matrix) ATP, but that the vectorial uptake of protons for the formation of water remains unaffected by the intraliposomal ATP/ADP ratio, since the RCR is only partly decreased at 100% intraliposomal ATP.

No stimulation of respiration by intraliposomal ADP was previously found with the reconstituted liver enzyme [13]. We therefore conclude that the described regulation of the proton pump by the ATP/ADP ratio is due to binding of ATP and/or ADP to the N-terminal matrix-oriented domain of subunit VIa-H, and thus occurs only in heart and skeletal muscle tissue. A scheme for regulation of the proton pump in muscle COX by nucleotides is presented in Fig. 6. The specific involvement in proton translocation has been shown by site-directed mutagenesis for the conserved amino acid Asp-135 in subunit I of *Escherichia coli* quinol oxidase [18] and Asp-132 in subunit I of *Rhodobacter sphaeroides* COX [19], which corresponds to Asp-91 in subunit I of COX from bovine [20]. The location of both, the evolutionary conserved aspartic acid in subunit I of COX and quinol oxidase, and of the nucleotide-binding 17 N-terminal amino acids of subunit VIa-H on the intracellular (bacteria) or corresponding matrix side (bovine [21]), suggest a direct interaction of bound nucleotides with the proton pore-controlling conserved aspartic acid in subunit I. The physiological function of turning off the proton pump in muscle COX at high matrix ATP/ADP ratios could be to stimulate thermogenesis in skeletal muscle at rest (e.g., during sleep).

5. Regulation of cytochrome c oxidase by other effectors

It was suggested that the large number of 10 nuclear coded subunits in mammalian COX, which are absent in the bacterial enzyme [3], have the function to regulate the activity via binding of allosteric effectors like substrates, cofactors, ions and hormones [22]. In a recent report we could demonstrate a direct interaction of diiodothyronines (3,3′-T_2 and 3,5-T_2) with isolated COX from bovine heart [23]. Maximal stimulation of activity (about 50%) was obtained with low concentrations (10^{-8} to 10^{-6} M) of 3,3′-T_2 at pH 6.4 and with 3,5-T_2 at pH 7.4. In contrast, 3,5,3′-triiodothyronine showed no or little stimulation of activity. Binding of the hormones to COX lead to conformational changes, as shown by changes in the visible

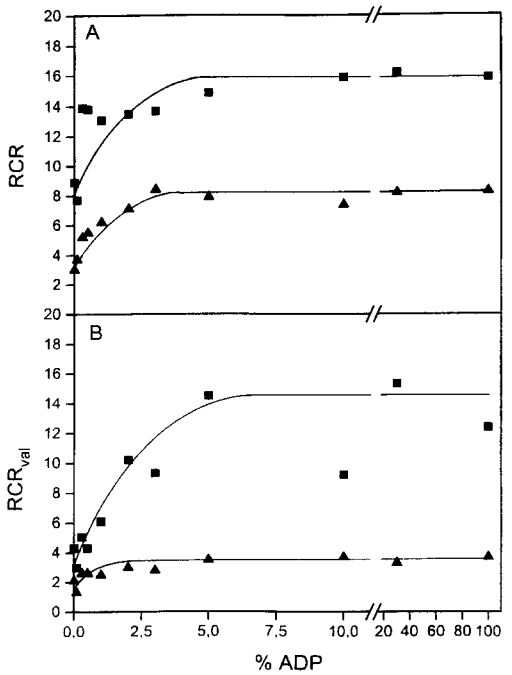

Fig. 5. Dependence of the respiratory control ratios RCR (A) and RCR$_{val}$ (B) of reconstituted COX on the intraliposomal concentrations of ATP and ADP. The total intraliposomal nucleotide concentration was held constant at 10 mM. The data were obtained at 5 μM (triangles) and 50 μM cytochrome *c* (squares) and the indicated percentage of intraliposomal ADP.

spectra of the oxidized enzyme. It is suggested that 'short-term' effects of thyroid hormones on mitochondrial respiration are at least partly due to allosteric interaction of diiodothyronines with the COX complex.

6. Disassembly of cytochrome c oxidase in Leigh's syndrome

Mitochondrial diseases are often associated with COX deficiency [24]. In many cases the COX deficiency is due to mutations or deletions of the mitochondrial DNA (mtDNA) [25], coding for COX subunits I–III. In other cases, like the Leigh syndrome, a nuclear-encoded protein was implicated, affecting the structure or the stability of the COX holoenzyme complex [26]. So far, in no case the molecular basis for a mitochondrial disease, based on nuclear mutations, has been established. From the changed kinetics of COX in fibroblasts from patients with Leigh's syndrome, a defective assembly of the enzyme complex was suggested [27,28].

We have investigated the properties of COX in fibroblasts of patients with Leigh's syndrome using immunological and kinetic methods [29]. ELISA titrations of mitochondria with monoclonal antibodies revealed a slight decrease of subunit II, intermediate decrease of subunit IV,

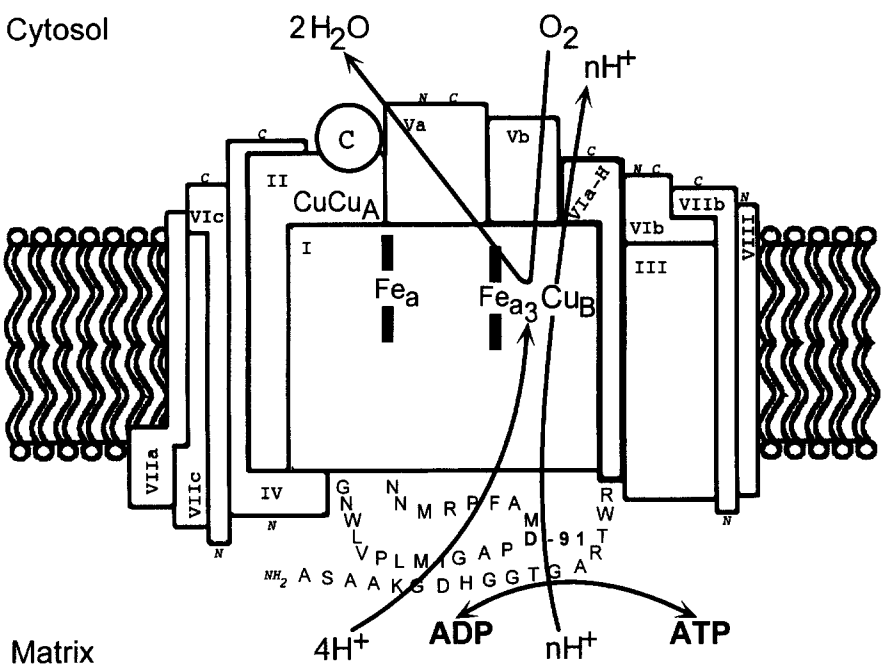

Fig. 6. Scheme of proposed regulation of the proton pump in COX from muscle. The 13 mostly transmembraneous (except subunits Va, Vb and VIb) subunits of mammalian COX are drawn by boxes with indicated cytosolic or matrix locations of their C- and N-termini, if known [3]. Asp-91 of bovine subunit I, located in a 21 amino acid loop between transmembraneous helices 2 and 3 on the matrix side [20], corresponds to Asp-135 of *E. coli* [18] and Asp-132 of *R. sphaeroides* [19], which both were shown to be involved in proton pumping. It is proposed that the 17 matrix-oriented N-terminal amino acids of subunit VIa-H, which were shown to bind the nucleotides [13], are in close contact with the loop of subunit I and control proton translocation according to the ATP/ADP ratio in the matrix. Cytochrome *c* is indicated by a large encircled C.

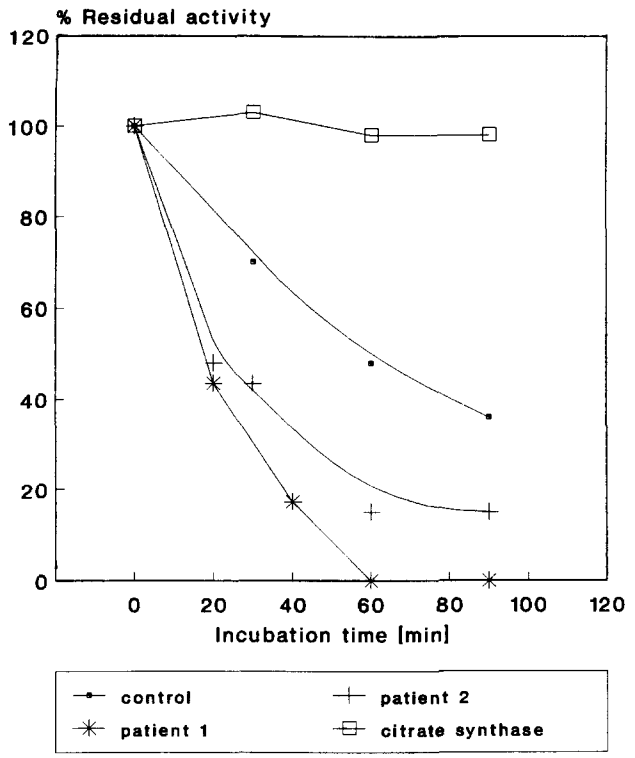

Fig. 7. Decreased thermostability of COX in cell extracts of fibroblast from patients with Leigh's syndrome. Cell lines from control and patients with Leigh's syndrome were obtained from Dr. C. Marsac, Paris and Dr. M.T. Zabot, Lyon. They were obtained from skin biopsy after informed consent. Fibroblasts were cultured according to standard procedures in RPMI 1640 medium with Glutamax I (Gibco BRL) supplemented with 10% fetal calf serum. Enzyme activities were determined in sonicated cell homogenates after pretreatment with digitonin as described [29]. COX activity was measured according to Cooperstein and Lazarow [31] in 0.1% dodecyl maltoside and citrate synthase according to Srere [32]. Cell extracts were incubated at 43° C, followed by enzyme activity determination at the indicated times. Citrate synthase activity (open squares) was the same for control and patient fibroblasts. COX activity of patient 1 (asterisks) showed 18%, patient 2 (crosses) 44% of control cells, but was set 100% at $t = 0$ min.

and strong decrease (to about 35% of control) of subunits Vab, VIac and VIIab of COX from patients. The biphasic change of activity of dodecyl maltoside-dissolved COX upon titration with anions (phosphate or guanidinium chloride); i.e., stimulation at low and inhibition at high anion concentrations, is lost with COX from patients. An immediate inhibition of activity was found instead [29]. In previous studies the biphasic change of activity upon titration of isolated COX from bovine heart with potassium chloride was converted into monophasic inhibition after selective removal of subunit VIb [30]. Therefore, the described results point to disassembly of the COX complex in patients with Leigh's syndrome. This conclusion is further demonstrated in the experiment shown in Fig. 7, indicating an increased temperature sensitivity of COX from patients with Leigh's syndrome. During incubation for 60 min at 43° C the activity of COX from control fibroblasts decreased to 50%, that of patient 1 (44% COX activity from control under standard conditions) to 20% and that of patient 2 (18% COX activity from control) to 0% of original activity.

The modulation of COX activity and proton pumping by nucleotides, as described above, is at least in part due to interaction with nuclear-coded subunits. Incomplete assembly of COX will therefore impair the regulatory properties of COX. Therefore mitochondrial diseases with partial COX deficiency could also be based on impaired regulatory functions of incomplete assembled holoenzyme.

Acknowledgements

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