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Regulation of Energy Transduction and Electron Transfer in Cytochrome *c* Oxidase by Adenine Nucleotides

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Cytochrome *c* oxidase from bovine heart contains seven high-affinity binding sites for ATP or ADP and three additional only for ADP. One binding site for ATP or ADP, located at the matrix-oriented domain of the heart-type subunit VIaH, increases the H^+/e^- stoichiometry of the enzyme from heart or skeletal muscle from 0.5 to 1.0 when bound ATP is exchanged by ADP. Two further binding sites for ATP or ADP, located at the cytosolic and the matrix domain of subunit IV, increases the K_M for cytochrome *c* and inhibit the respiratory activity at high ATP/ADP ratios, respectively. We propose that thermogenesis in mammals is related to subunit VIaL of cytochrome *c* oxidase with a H^+/e^- stoichiometry of 0.5 compared to 1.0 in the enzyme from bacteria or ectotherm animals. This hypothesis is supported by the lack of subunit VIa isoforms in cytochrome *c* oxidase from fish.

KEY WORDS: Cytochrome *c* oxidase; H^+/e^- stoichiometry; thermogenesis; adenine nucleotides; regulation of activity; ectotherm animals; ATP/ADP ratios; allosteric enzyme; monoclonal antibodies; rate of respiration.

INTRODUCTION

The reduction of oxygen for the generation of metabolic energy (ATP) is catalyzed in aerobic organisms by quinol or cytochrome oxidases, all of which contain evolutionary conserved structures (Chepuri *et al.*, 1990), leading to a similar vicinity of the binuclear oxygen-binding center, composed of a heme group (heme *o* or a_3) and Cu_B (for review see Saraste, 1990; Trumpower and Gennis, 1994). With increasing complexity of the organism the subunit composition of cytochrome oxidases increased, containing 1–4 subunits in bacteria (Saraste, 1990) and 7–13 subunits in eukaryotes (Kadenbach *et. al.*, 1986; 1987; Capaldi, 1990). Three "catalytic" subunits of the eukaryotic enzyme are encoded on mitochondrial DNA, the "supernumerary" subunits, suggested to have a regulatory function (Kadenbach, 1983; 1986, Poyton *et al.*, 1988), are encoded in nuclear DNA. The number of 13 nuclear coded subunits in mammalian cytochrome *c* oxidase remained for a long time a matter of discussion (Kadenbach *et al.*, 1987), but was finally confirmed by the crystal structure of the dimeric enzyme from bovine heart (Tsukihara *et al.*, 1995, 1996). The crystal structure of cytochrome *c* oxidase from *Paracoccus denitrificans* revealed four subunits (Iwata *et al.*, 1995). Thirteen subunits were also identified in cytochrome *c* oxidase from birds (e.g., turkey; see Kadenbach *et al.*, 1997) and fish (e.g., tuna; see Arnold *et al.*, 1997), but eleven subunits were identified in yeast (Geier *et. al.*, 1994) and only seven in *Dictyostelium discoideum* (Bisson and Schiavo, 1986). This review summarizes recent data on the regulatory function of nuclear coded subunits.

ISOFORMS OF CYTOCHROME *C* OXIDASE

In addition to the large number of subunits, eukaryotic cytochrome *c* oxidases are also characterized by

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the expression of partly tissue-specific isoforms of nuclear coded subunits. These isoforms replace corresponding subunits leading to different isozyme complexes with a constant number of subunits, e.g., 13 in mammals. In the slime mold *Dictyostelium discoideum*, subunit VII, related to mammalian subunit VIc (Capaldi *et al.*, 1990), occurs in two different isoforms (Bisson and Schiavo, 1986), which are differently expressed depending on the environmental oxygen concentration (Bisson *et al.*, 1997). In yeast, subunit V, corresponding to mammalian subunit IV, occurs in two different isoforms (Va and Vb, Cumsky *et al.*, 1987), which are alternatively expressed, depending also on the environmental oxygen concentration (Poyton *et al.*, 1988). The two isozymes differ in their kinetic properties (Waterland *et al.*, 1991; Allen *et al.*, 1995).

In mammals two different isoforms were observed for subunits VIa, VIIa, and VIII in most species, but in human only one isoform of subunit VIII, and in rat a single isoform of subunit VIIa was identified (for reviews see Kadenbach and Reimann, 1992; Grossman and Lomax, 1997). Recently a new isoform of subunit VIIa, which is expressed in all tissues, was identified in mouse (Segade *et al.*, 1996). The mature protein shows only 50% identity with rat liver subunit VIIa, while the mature proteins of subunits VIIaL and VIIaH from bovine are 72% identical. In rainbow trout two isoforms of subunit Vb were found, being expressed in heart and liver at different ratios (Freund and Kadenbach, 1994). In tuna new isoforms were identified for subunit Va, VIc, and VIIb (see below).

BINDING OF ATP AND ADP TO CYTOCHROME *C* OXIDASE

Isolated cytochrome *c* oxidase from bovine heart binds seven molecules of ATP and ten molecules of ADP per monomer with average dissociation constants K_d of 15 and 28 μM, respectively. This was shown by equilibrium dialysis with [^{35}S]ATPαS or [^{35}S]ADPαS for 72 h (Napiwotzki *et al.*, 1997). In a previous study the enzyme from bovine liver was found to bind six molecules of ATP, and the two-subunit enzyme from *Paracoccus denitrificans* was found to bind one molecule of ATP per monomer, also by using the equilibrium dialysis method (Rieger *et al.*, 1995). Competition studies with cold ADP or ATP, present during equilibrium dialysis with [^{35}S]ATPαS or [^{35}S]ADPαS, respectively, demonstrated that all seven binding sites for ATP at cytochrome *c* oxidase from bovine heart can be exchanged by ADP, while only seven of the ten binding sites for ADP can be exchanged by ATP (Napiwotzki and Kadenbach, 1998). These results demonstrate that the seven binding sites for ATP in the bovine heart enzyme bind also ADP, and three additional sites bind only ADP. In living cells the concentrations of ATP and ADP are in the millimolar range (Schwenke *et al.*, 1981). Therefore we conclude that *in vivo* all binding sites for adenine nucleotides at cytochrome *c* oxidase are occupied by ATP or ADP, depending on the cytosolic and/or mitochondrial ATP/ADP ratio.

In contrast to the rapid exchange of bound ADP by ATP (shown by kinetic studies; Napiwotzki and Kadenbach, 1998), their binding to the cholate containing isolated enzyme, purified by using cholate as detergent, is a slow process taking hours. This was shown by binding studies using a filtration method, and was explained by conformational changes of the enzyme upon binding of cholate at the ADP (or ATP) binding sites (Napiwotzki *et al.*, 1997). In crystals of cytochrome *c* oxidase from bovine heart ten molecules of cholate per enzyme monomer were identified by using radioactive cholate (Napiwotzki *et al.*, 1997). Two molecules of cholate, which is structurally very similar to ADP, have been located in the crystal structure of the enzyme, one at the N-terminal domain of subunit VIa, the other between subunits I and III (Tsukihara *et al.*, 1996).

Although the average dissociation constant determined for ADP is higher than for ATP, the binding site at subunit VIaH (heart type), which modifies the H^+/e^- stoichiometry, and at subunit IV, which regulates the rate of electron transfer, have a higher affinity to ADP than to ATP (see below). This suggests that regulation of the H^+/e^- stoichiometry and the rate of respiration of cytochrome *c* oxidase occurs at very high cellular ATP/ADP ratios.

ADP CAUSES A SPECTRAL CHANGE OF ISOLATED CYTOCHROME *C* OXIDASE

Preincubation of isolated cytochrome *c* oxidase from bovine heart with increasing concentrations of ADP for 24 h at 0°C changed the oxidized visible spectrum (Napiwotzki *et al.*, 1997). The binding of ADP decreased the absorbance of the γ-band maximally to about 88% and the α-band to 94% of the absorbance of the untreated enzyme. The spectral

changes are independent of the presence of ATP. Half-maximal decrease of absorbance is obtained at 5 μM ADP. In contrast to ADP, other nucleotide diphosphates like GDP, CDP, and UDP have no significant effect on the oxidized visible spectrum of cytochrome *c* oxidase. Only preincubation with IDP, which is structurally similar to ADP, induces changes in the visible spectrum of the enzyme. The maximal spectral changes with IDP are about 50% of those obtained with ADP; and half-maximal spectral changes were obtained at about 10-fold higher concentrations of IDP than ADP. This indicates a lower affinity for IDP and a high specificity for the adenine moiety in the ADP binding site. The subunit location of this ADP binding site is not known, but it is different from the nucleotide binding sites at subunit VIaH, changing the H^+/e^- stoichiometry (Frank and Kadenbach, 1996), and at subunit IV, changing the rate of ferrocytochrome *c* oxidation (see below). This follows from the lack of effects of preincubation of the enzyme with monoclonal antibodies to subunits VIaH+VIc and to subunit IV on the spectral changes obtained with the heart and liver enzyme (Napiwotzki *et al.*, 1997). When incubations with ADP were performed under identical conditions except for the prresence of dodecylmaltoside instead of Tween 20 or Tween 80, the spectral changes were completely prevented, suggesting perturbation of regulatory properties of cytochrome *c* oxidase by dodecylmaltoside.

INFLUENCE OF ATP ON THE H^+/e^- STOICHIOMETRY OF THE HEART ISOZYME

High intraliposomal ATP/ADP ratios decrease the respiratory control ratio (RCR) (Rohdich and Kadenbach, 1993; Kadenbach *et al.*, 1995) and the H^+/e^- stoichiometry of reconstituted cytochrome *c* oxidase from bovine heart, but not from bovine liver, by about 50% (Frank and Kadenbach, 1996). In previous studies it was shown that ADP binds to the N-terminal, matrix-oriented domain of subunit VIaH, by using a monoclonal antibody reacting with subunit VIaH (heart-type) and subunit VIc (Anthony *et al.*, 1993). Preincubation of the enzyme with the monoclonal antibody before reconstitution (Schneyder *et al.*, 1991) prevents the decrease of H^+/e^- stoichiometry. Half-maximal decrease of H^+/e^- stoichiometry was found at 99% ATP, with [ATP + ADP] = 5 mM (Frank and Kadenbach, 1996). Therefore only at very high ATP/ADP ratios in the matrix is the H^+/e^- stoichiometry decreased to the value of 0.5, compared to 1.0 measured at lower ATP/ADP ratios. The H^+/e^- stoichiometry of reconstituted cytochrome *c* oxidase from bovine liver is not influenced by the intraliposomal ATP/ADP ratio (Kadenbach *et al.*, 1997). We have found that various preparations of cytochrome *c* oxidase from bovine liver and bovine kidney, containing the liver type of subunit VIa (VIaL), showed after reconstitution in liposomes H^+/e^- stoichiometries of maximally 0.5, in contrast to H^+/e^- stoichiometries of close to 1.0 measured with various preparations of the enzyme from bovine heart (Frank and Kadenbach, in preparation). This result could indicate an intrinsic difference in the maximal H^+/e^- stoichiometry of cytochrome *c* oxidase containing subunit VIaL or VIaH.

Cytochrome *c* oxidase of adult rat heart contains one third of the liver type and two thirds of the heart type of subunit VIa (Kadenbach *et al.*, 1990), while almost 100% of the heart type of subunit VIa is expressed in skeletal muscle (Anthony *et al.*, 1990). In cytochrome *c* oxidase of fetal heart and fetal skeletal muscle from human (Bonne *et al.*, 1993) and mouse (Parsons *et al.*, 1996) the liver-type subunit VIaL is predominantly expressed. After birth a switch to the heart-type subunit VIaH occurs partly in heart and almost completely in skeletal muscle. This result further supports the postulate that subunit VIaH contributes to optimal efficiency of energy transduction in cytochrome *c* oxidase of muscle tissue at work load, and to participate in thermogenesis at rest or high ATP/ADP ratios (Rohdich and Kadenbach, 1993; Kadenbach *et al.*, 1995).

CYTOCHROME *C* OXIDASE FROM TUNA OCCURS IN VARIOUS ISOZYMES

Cytochrome *c* oxidase was isolated from tuna heart and liver using Triton X-114, Triton X-100, and cholate as detergents (Kadenbach *et al.*, 1986). The purified enzymes showed 13 polypeptide bands on SDS-PAGE, with a triplet in the region of subunit Va+Vb, but lacking subunit VIa (Arnold *et al.*, 1997). The absence of subunit VIa was previously also described for isolated cytochrome *c* oxidases from rainbow trout heart and liver (Freund and Kadenbach, 1994). In a later study, however, subunit VIa was identified in an enzyme preparation from carp heart, and the corresponding cDNAs for subunit VIa from carp heart and trout liver were cloned and characterized (Hüttemann *et al.*, 1997). In order to obtain the com-

plete subunit composition of cytochrome *c* oxidases from tuna heart and liver the enzymes were immunoprecipitated with a monospecific but polyclonal antibody to subunit IV from dodecylmaltoside-solubilized mitochondria. The immunoprecipitates revealed a clear band corresponding to subunit VIa, and a new band in the region between subunit VIb and VIc of the heart enzyme (subunit VIc-2).

The polypeptides of the enzymes from tuna heart and liver were separated by SDS-PAGE, and the N-terminal and/or internal amino acid sequences of blotted or excised gel bands were determined. By comparison with sequences of subunits from mammalian enzymes 13 different subunits were identified, corresponding to those of the mammalian enzyme. Two isoforms were identified each for subunit Va in heart and liver, for subunit VIc only in heart, for subunit VIIb either in heart or liver (tissue-specific), and for subunit VIII only in liver. No isoforms could be found for subunits VIa and VIIa, both of which are expressed in mammals in two different isoforms (Linder *et al.*, 1995). Isoforms for subunits Va, VIc, and VIIb have not yet been described in other species. However, isoforms of subunits Vb and VIIa, described for cytochrome *c* oxidase from rainbow trout heart and/or liver (Freund and Kadenbach, 1994), could not be found in the enzymes from tuna heart and liver. These results indicate highly species-specific expression of isoforms for almost all nuclear coded subunits of eukaryotic cytochrome *c* oxidases.

ATP IS AN ALLOSTERIC INHIBITOR OF CYTOCHROME *C* OXIDASE

The activity of isolated cytochrome *c* oxidase from bovine heart is hardly influenced by added ATP or ADP, due to the slow exchange of bound cholate by the nucleotides. After preincubation of the enzyme for 24 h at 0°C with the nucleotides in phosphate buffer and 1% Tween 20 (or Tween 80), however, the rate of polarographically measured ascorbate oxidation is about 50% lower with ATP than with ADP (Napiwotzki *et al.*, 1997). In addition, the K_M for cytochrome *c* is higher in the presence of ATP than of ADP. In contrast, preincubation of the enzyme with the nucleotides in 0.5% dodecylmaltoside results in about doubling of activity, but no inhibition by ATP is observed. Dodecylmaltoside was shown to remove selectively subunit VIb from the enzyme complex accompanied by stimulation of activity (Weishaupt and Kadenbach, 1992). Preincubation of the enzyme with a monoclonal antibody to subunit IV (Schneyder *et al.*, 1991) relieves the inhibition by ATP, indicating binding of ATP (or ADP) to subunit IV. Measurement of the activity at increasing ATP/ADP ratios showed half-maximal inhibition at 99% ATP, when the concentration of the sum of both nucleotides was constant ([ATP+ADP] = 5 mM, Napiwotzki *et al.*, 1997).

When the bovine heart enzyme was reconstituted in liposomes the K_M for cytochrome *c* of the controlled ascorbate respiration (respiratory control ratio, RCR ~ 8) was about 6-fold higher in the presence of extraliposomal ATP than ADP (7 μM instead of 1 μM), independent of the presence of intraliposomal ATP or ADP (Napiwotzki and Kadenbach, 1998). Preincubation of the reconstituted enzyme (proteoliposomes) with the monoclonal antibody to subunit IV increased the v_{max} of controlled respiration (RCR ~ 6) by 50% (TN ~ 35), and resulted in the same K_M for cytochrome *c* (5 μM) in the presence of extraliposomal ATP as well as ADP. This result demonstrates binding of the monoclonal antibody to the cytosolic domain of subunit IV. From these results we conclude that the C-terminal, cytosol-oriented domain of subunit IV contains a binding site for ATP or ADP which increases the K_M for cytochrome *c* upon exchanging bound ADP by ATP.

With increasing cytochrome *c* concentrations the activity/substrate concentration (*v/s*) relationship of the isolated bovine heart enzyme showed saturation curves in the presence of both nucleotides (Napiwotzki *et al.*, 1997). Also with the reconstituted enzyme saturation kinetics were measured independent of the presence of ATP or ADP inside or outside of the proteoliposomes. In order to demonstrate that inhibition of cytochrome c oxidase activity by ATP represents a feature of all eukaryotic enzymes, we investigated also the effect of nucleotides on the enzymes from tuna heart and liver. The isolated enzymes were missing some subunits (Arnold *et al.*, 1997), therefore the activity of cytochrome *c* oxidase was measured in Tween 20-solubilized mitochondria from tuna heart and liver (Arnold and Kadenbach, 1997). Since soluble extracts of mitochondria contain ATPases, we included an ATP-regenerating system consisting of PEP (phosphoenolpyruvate) and PK (pyruvate kinase). The polarographically measured ascorbate respiration revealed saturation kinetics in the presence of 5 mM ADP and of only 5 mM ATP, but the addition of the ATP-regenerating system resulted in a sigmoidal *v/s* relationship with complete inhibition of ascorbate respiration up

to 6 μM cytochrome *c*. It should be mentioned that the K_M of cytochrome *c* oxidase in Keilin-Hartree particles (in the presence of TMPD) was determined to 0.1 and 1 μM for the high- and low-affinity phase of cytochrome *c* oxidation, respectively (Ferguson-Miller *et al.*, 1976). The switch from hyperbolic to sigmoidal *v/s* curves occurs apparently at very high ATP/ADP ratios. This was further demonstrated by measuring the *v/s* curves at variable concentrations of PK, resulting in different steady state levels of the ATP/ADP ratio. A family of curves was obtained with no sigmoidicity in the absence of PK and strong sigmoidal curvature at high PK concentrations. The Hill plot of the data revealed a Hill coefficient of 2.0 at the highest PK concentration, indicating cooperativity of two functional binding sites for cytochrome *c*.

The cooperativity of the enzyme in the presence of high ATP/ADP ratios is lost when the activity is measured with TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine), which was assumed to mediate electron transfer from ascorbate to enzyme-bound cytochrome *c*, thus omitting the dissociation of the substrate (Ferguson-Miller *et al.*, 1978). Cooperativity involves binding and dissociation of the substrate, and therefore no sigmoidal *v/s* curves will be obtained. Also in the presence of dodecylmaltoside no sigmoidal *v/s* curves were obtained at high ATP/ADP ratios (Arnold and Kadenbach, unpublished results). Dodecylmaltoside was shown to dissociate the dimeric enzyme into monomers (Suarez *et al.*, 1984; Bolli *et al.*, 1985; Hakvoort *et al.*,b 1987) and would prevent cooperative interactions of two cytochrome *c* binding sites at the monomers in the dimeric enzyme complex.

Allosteric inhibition of cytochrome *c* oxidase is apparently due to binding of ATP to the matrix domain of the transmembranous subunit IV, which has a dumbbell like structure (Tsukihara *et al.*, 1996). This was shown by relief of the sigmoidal inhibition curve after preincubation of solubilized mitochondria from tuna heart with the monoclonal antibody to subunit IV, and by measurements with submitochondrial particles. Only when ATP and the ATP-regenerating system were inside the vesicles was a sigmoidal inhibition curve obtained (Arnold and Kadenbach, 1997).

The cooperativity of two cytochrome *c* binding sites in cytochrome *c* oxidase requires essentially cardiolipin, as could be shown with the reconstituted enzyme from bovine heart. Only when 5% cardiolipin was included during reconstitution of the enzyme with asolectin was a sigmoidal inhibition curve measured at high intraliposomal ATP/ADP ratios with increasing concentrations of cytochrome *c*. Cardiolipin was also shown to stimulate the activity of the mitochondrial phosphate carrier (Kadenbach *et al.*, 1992) and of other mitochondrial carriers, which are all assumed to function as dimers (Palmieri *et al.*, 1993). From these results we suggest that cooperative inhibition of cytochrome *c* oxidase at high ATP/ADP ratios is due to binding of ATP to the matrix domain of subunit IV and to conformational interactions between the two monomers in the dimeric enzyme complex, resulting in a positive cooperativity of the two cytochrome *c* binding sites.

BINDING SITES FOR ATP AND/OR ADP IN CYTOCHROME *C* OXIDASE FROM BOVINE HEART

From results in the literature and from the data presented above the subunit locations of five of the ten adenine nucleotide binding sites (seven for ATP or ADP and three only for ADP) in cytochrome *c* oxidase from bovine heart can be defined. To make the locations graphically clear we indicated them in a model of dimeric cytochrome *c* oxidase, drawn according to the crystal structure of the bovine heart enzyme (Tsukihara *et al.*, 1996), as shown in Fig. 1. One binding site for ATP or ADP is located at the N-terminal domain of subunit VIaH (heart type), which was verified in the crystal structure of the enzyme by a bound cholate molecule, which is structurally very similar to ADP (Tsukihara *et al.*, 1996). This nucleotide binding site is involved in tissue-specific regulation of H^+/e^- stoichiometry (Frank and Kadenbach, 1996). Another binding site for ATP or ADP was identified at the C-terminal domain of subunit VIa of cytochrome *c* oxidase from bovine heart, bovine liver, and yeast by labeling with radioactive 2-azido-ATP (Taanman *et al.*, 1994). The second cholate molecule, identified in the crystal structure between the catalytic subunits I and III, indicates a third binding site for ADP (Tsukihara *et al.*, 1996). This binding site could be responsible for the spectral changes of the enzyme by ADP, as described above. Two further binding sites for ATP or ADP are located at subunit IV which affect the kinetics of ferrocytochrome *c* oxidation: one at the C-terminal, cytosol-oriented domain, another at the N-terminal, matrix-oriented domain. Subunit IV was previously photoaffinity-labeled with [γ-^{32}P]ATP (Bisson *et al.*, 1987) and 8-azido-[γ-^{32}P]ATP (Rieger *et al.*, 1995) at the isolated enzyme, and with 8-azido-

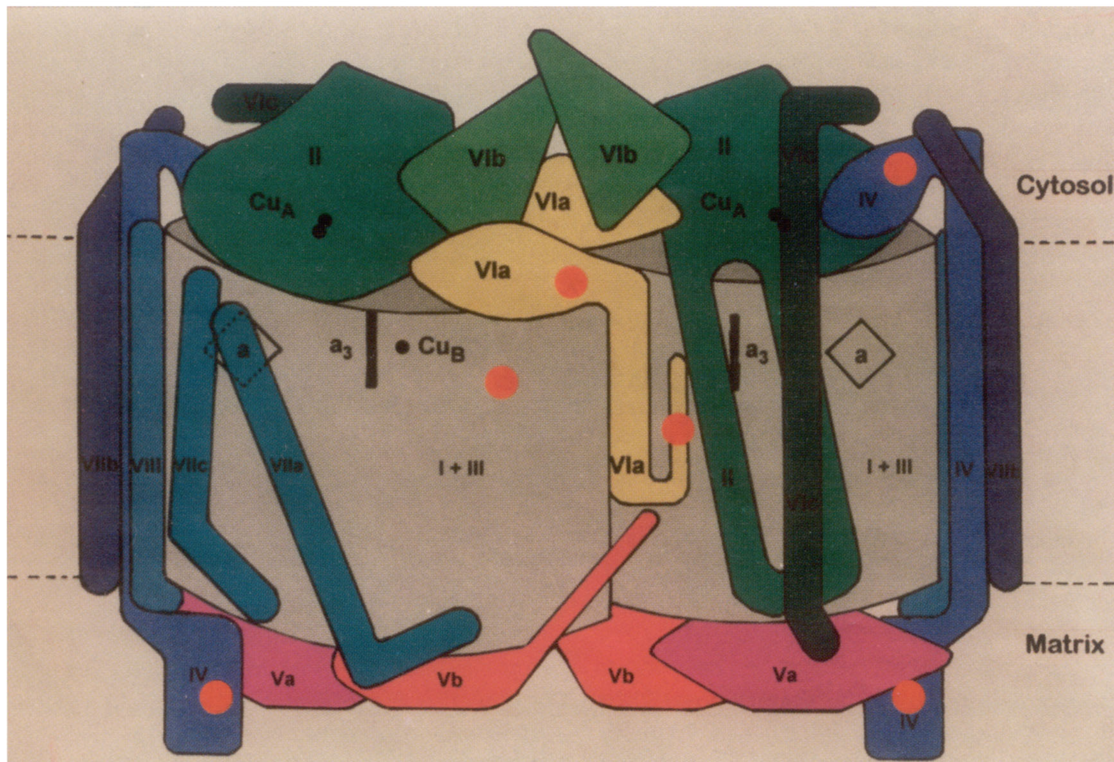

Fig. 1. Scheme of the dimeric cytochrome *c* oxidase complex, based on the crystal structure of the enzyme from bovine heart (Tsukihara *et al.*, 1996). The transmembranous helices of subunits IV and VIII are 50° tilted (not visible from this view). The membrane is indicated by dotted lines, with the upper side representing the cytosolic, the lower side the matrix phase. The hemes *a* (*a*) and a_3 (a_3), located perpendicular to each other, are indicated by rhomboids and black bars, respectively, the two copper atoms of Cu_A and the one of Cu_B are indicated by black dots. The postulated subunit location of five ATP and/or ADP binding sites (one between subunits I and III, two at subunit IV, accessible from the cytosolic or matrix side, and two at subunit VIa, accessible from the cytosolic or matrix side) are indicated by orange circles.

[γ-*32*P]ATP at the reconstituted enzyme (Reimann *et al.*, 1988). The latter labeling suggested the cytosolic location of an ATP binding site at subunit IV.

One or two additional binding sites for ATP or ADP are located at subunits VIIabc, as concluded from labeling studies with 8-azido-ATP (Bisson *et al.*, 1987; Reimann *et al.*, 1988; Rieger *et al.*, 1995).

NO ISOFORMS FOR SUBUNIT VIa OCCUR IN CYTOCHROME *C* OXIDASES FROM FISH

In all mammals two different isoforms of subunit VIa are expressed, subunit VIaH in heart or skeletal muscle, and subunit VIaL in all other tissues (Linder *et al.*, 1995). In order to investigate the hypothesis that thermogenesis in mammals is associated with a switch of H^+/e^- stoichiometry of the muscle isozyme from 0.5 at rest (high ATP/ADP ratios) to 1.0 at work (lower ATP/ADP ratios), we investigated the possible expression of subunit VIa isoforms in ectotherm fish. In previous studies no subunit VIa was found in isolated cytochrome *c* oxidases from rainbow trout heart and liver (Freund *et al.*, 1994), which was apparently due to loss of this subunit during isolation. In later studies subunit VIa was found in the isolated enzyme from carp heart, and with RT-PCR using primers deduced from an internal amino acid sequence of this subunit the complete cDNA of subunit VIa from carp heart and rainbow trout liver could be determined (Hüttemann *et al.*, 1997). The deduced amino acid sequences of the two mature proteins showed 82% identity among each other, but only 50–60% identity to mammalian subunits VIaH as well as VIaL, corresponding to the low percentage identity (50–60%) between the mammalian subunits VIaH and VIaL. A Northern blot with mRNA from liver, heart, and white skeletal muscle from carp

revealed under low stringent washing conditions only a single band (Hüttemann and Kadenbach, unpublished results). Finally, half-quantitative PCR with total RNA from rainbow trout liver, heart, spleen, skeletal muscle, and gill with gene-specific primers for trout subunits Va and VIa revealed a constant ratio of amplified amounts of DNA products for subunit Va and VIa (Exner, 1997). These results were taken to indicate the absence of isoforms for subunit VIa in ectotherm fish. A phylogenetic tree was constructed, based on the assumption of 75 My distance between liver and heart isoforms of subunit VIa from rat and human (Saccone *et al.*, 1991), indicating independent evolution of subunits VIaH and VIa from fish for 265 My, and 340 My for the time of gene duplication of subunit VIaL and subunits VIaH + VIa from fish (Exner, 1997).

ROLE OF CYTOCHROME *C* OXIDASE IN THERMOGENESIS (HYPOTHESIS)

From the above results we propose that the evolution of thermogenesis in mammals was accompanied by the expression of the liver-type isoform of subunit VIa of cytochrome *c* oxidase, which has a H^+/e^- stoichiometry of 0.5 as compared to 1.0 in the enzyme from bacteria and ectotherm animals. In heart and skeletal muscle a tissue-specific isoform of subunit VIa (VIaH) is expressed in order to optimize the efficiency of energy transduction during work. This subunit contains at the N-terminal, matrix-oriented domain a binding site for ADP or ATP, which upon exchanging bound ATP by ADP increases the H^+/e^- stoichiometry from 0.5 to 1.0. This exchange occurs during work, when the ATP/ADP ratio in the mitochondrial matrix decreases. Half-maximal decrease occurs when 99% of the sum of free [ATP+ADP] is ATP. It should be noted that about 95% of cellular ADP is protein-bound and does not contribute to the concentration of free ADP (Veech *et al.*, 1979). In fact the free ATP/ADP ratio in rat heart was determined to 200–1000 (From *et al.*, 1990), although the total mitochondrial and cytosolic ATP/ADP ratios were determined in rat liver *in vivo* to 0.9 and 5.9, respectively (Schwenke *et al.*, 1981). We suppose that during rest (no work performance) the ATP/ADP ratio of free nucleotides in skeletal muscle mitochondria increases above 95% ATP, accompanied by the exchange of bound ADP at subunit VIaH by ATP and decrease of H^+/e^- stoichiometry to 0.5. We further propose that isozymes of cytochrome *c* oxidase, containing the liver type of subunit VIa, which are expressed in all tissues including smooth muscle (Anthony *et al.*, 1990) but not in heart and skeletal muscle (Linder *et al.*, 1995), exert a H^+/e^- stoichiometry of 0.5. This lower H^+/e^- stoichiometry of cytochrome *c* oxidase would contribute to nonshivering thermogenesis in mammals, because part of the free energy of oxygen reduction will be released as heat, instead of a proton gradient which can be used for the synthesis of ATP. At rest the skeletal muscle tissue, comprising the largest part of mammalian body weight, participates in thermogenesis, but at work cytochrome *c* oxidase from heart and skeletal muscle changes to higher efficiency of energy transduction and thus reduce the amount of metabolic heat.

It is interesting to note that different isoforms of subunit VIa occur also in warm-blooded birds. In turkey different N-terminal amino acid sequences were determined in subunits VIa from liver and skeletal muscle. In heart, however, the same sequence was found as in liver (Kadenbach *et al.*, 1997). These results are in accordance with the view that endothermy was associated with the evolution of the liver type of subunit VIa, inducing a lower H^+/e^- stoichiometry of cytochrome *c* oxidase, compared with the enzyme from bacteria and ectotherms.

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