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Research Article

The possible role of isoforms of cytochrome c oxidase subunit VIa in mammalian thermogenesis

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Abstract. A single cDNA of cytochrome c oxidase subunit VIa was characterised from liver, heart and the thermogenic organ of the partially endotherm tuna fish. The amino acid sequence revealed high identity with subunit VIa from carp and trout, but low identity to subunits VIaL (liver type) and VIaH (heart type) of mammalian cytochrome c oxidase. In reconstituted cytochrome c oxidase from bovine heart, the H^+/e^- stoichiometry is decreased from 1.0 to 0.5 at high intraliposomal ATP/ADP ratios via exchange of bound ADP by ATP at the matrix domain of the transmembraneous subunit VIaH. Reconstituted cytochrome c oxidase from bovine liver and kidney, containing subunit VIaL, revealed H^+/e^- ratios below 0.5, independent of the ATP/ADP ratio. The results suggest the evolution of three types of subunit VIa. Subunits VIaH and VIaL are postulated to participate in mammalian thermogenesis.

Key words. Cytochrome c oxidase; H^+/e^- stoichiometry; evolution; mammalian thermogenesis; fish tissues; homeothermy; efficiency of energy transduction.

Introduction

The molecular basis of non-shivering thermogenesis in mammals is not fully understood [1, 2]. In rodents, a special organ has evolved, the brown adipose tissue, where thermogenesis is hormonally induced and based on the conversion of the mitochondrial proton motive force into heat via the uncoupling protein [3–5]. Free fatty acids are assumed to open the proton channel of the uncoupling protein [6, 7]. Larger mammals, including human, have only negligible amounts of brown adipose tissue and the uncoupling protein (UCP-1) and thus require a different mechanism for non-shivering thermogenesis. Two further recently described uncoupling protein, UCP-2 [8] and UCP-3 [9], could participate in non-shivering thermogenesis in mammals, but their contribution remains to be established.

In mitochondria from endotherm animals, a significantly higher rate of respiration in the absence of ADP has been measured compared to that of mitochondria from ectotherms [10]. Brand [2] postulated that proton leakage across the inner mitochondrial membrane could account for heat production in endotherm animals. The proton permeability of liver mitochondria from rat and a similar-sized reptile, which has a seven-fold lower standard metabolic rate, was compared [11]. The proton leak in the reptile was four to five times lower, suggested to be due to a different lipid composition of the inner mitochondrial membranes. In mitochondrial membranes of the liver of large and small mammals, however, differing in mass-specific metabolic rate by a factor of 10 [12], only a weak correlation between proton flux per square centimetre and phospholipid composition of mitochondrial membranes was found [13].

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Recently, variable efficiency of energy transduction in mitochondria from heart and skeletal muscle was described, based on different H^+/e^- ratios of cytochrome c oxidase. At high intraliposomal ATP/ADP ratios (half-maximal at ATP/ADP = 100), the H^+/e^- ratio of the reconstituted bovine heart enzyme decreased from 1.0 to 0.5 [14]. ATP and ADP were shown to interact with the matrix domain of subunit VIaH [15, 16]. It was suggested that this mechanism participates in thermogenesis in heart and skeletal muscle at rest, i.e. when the matrix ATP/ADP ratio is high [17, 18].

Cytochrome c oxidase from mammals contains three mitochondrial-coded and ten nuclear-coded subunits, which are partly expressed in tissue-specifc isoforms [19]. The heart type of subunit VIa (VIaH) is expressed in heart and skeletal muscle [20], but not in smooth muscle [21], whereas the liver type (VIaL) is expressed in all other tissues. The mature amino acid sequences of the two isoforms are only 55% (bovine, rat) and 61% (human) identical. In liver and heart from carp and trout, only one isoform of subunit VIa was found, revealing low amino acid sequence identity with mammalian subunits VIaH and VIaL [22]. The absence of subunit VIa isoforms in ectotherm fishes was correlated with the lack of thermogenesis in these animals. The tuna fish belongs to the group of partially endotherm fishes, with a temperature above water temperature in muscle, viscera, brain and eye [23–25]. Therefore it was of interest to investigate if two different isoforms of subunit VIa are expressed in tuna, as in mammals.

Here we present the cDNA sequence of cytochrome c oxidase subunit VIa from tuna, which is homologous to subunit VIa from carp and trout, suggesting a different mechanism of thermogenesis between tuna and mammals. Evolutionary analysis indicates a third type of subunit VIa in cytochrome c oxidase from fishes, more distantly related to the mammalian liver-type than to the heart-type isoform. Whereas the H^+/e^- ratio of reconstituted cytochrome c oxidase from bovine heart decreases from 1.0 to 0.5 at high intraliposomal ATP/ADP ratios, the H^+/e^- ratio of the enzyme from liver and kidney was below 0.5, independent of the intraliposomal ATP/ADP ratio. It is postulated that subunits VIaH and VIaL could participate in mammalian thermogenesis by decreasing the H^+/e^- ratios of the isozymes, accompanied by increased respiration and heat production.

Materials and methods

Cloning and sequencing of tuna subunit VIa. Total RNA was prepared from heart, liver, skeletal muscle and heater organ tissue from tuna by phenol/chloroform extraction [26]. Total cDNA first strands were generated through reverse transcription which was primed with dT_{17}-tailed oligonucleotide (QT-primer) containing two appended primer sequences (Q_{Inner} and Q_{Outer}) according to Frohman [27]. 3′-RACE (rapid amplification of cDNA ends) was carried out as a touch-down PCR with the degenerated gene-specific primer P1 (5′-TNGCNYTNCCNDSNGTNG-3′) derived from a conserved amino acid region of mammalian liver/heart isoforms and of carp/rainbow trout subunit VIa (fig. 1) and the Q_{Outer}-primer which corresponds to the 5′-terminal sequence of the QT-primer. For all PCR amplifications, the Expand™ High Fidelity PCR System (Boehringer Mannheim) was used. Thirty cycles were used in all reactions in which the annealing temperature of the initial cycles was reduced by 2 °C every two cycles: denaturation for 60 s at 94 °C; annealing for 45 s at 40–50 °C; elongation for 40 s at 72 °C. For further specification, a nested PCR was carried out with the degenerated primer P2 (5′-TGGGNGAYGGNAAYCA-3′) derived from a second conserved amino acid region of subunit VIa (fig. 1) and primer Q_{Inner} using 1 µl of a 1:100 dilution of the previous PCR product as template. The final PCR product was cloned into pBluescript SK^- and sequenced in both directions with the ^{T7}Sequencing™ Kit (Pharmacia Biotech). From the obtained 3′ cDNA sequence, two gene-specific reverse primers were designed (P3, P4) for 5′-RACE-PCR [27] as depicted in figure 1. 5′ reverse transcription was carried out with the outer gene-specific primer P4 followed by RNaseH treatment. A polyA tail was appended to the cDNA first strands with terminal desoxynucleotidyl transferase (Amersham). cDNA second strands were generated with the QT-primer. PCR amplification was carried out with primers P4 and Q_{Outer} (denaturation for 60 s at 94 °C; annealing for 45 s at 56 °C; elongation for 50 s at 72 °C). Nested PCR was carried out as described above using the primers P3 and Q_{Inner}. The PCR product was cloned and sequenced as described above.

Screening for subunit VIa isoforms in the four tuna tissues was performed by 3′-RACE-PCR, including nested PCR, with the following four different degenerated primers of cDNA regions where the protein sequence is homologous for all studied tissues and species: NH(T/S)LFHN, P3 (5′-AAYCAYWSNYTNTTYCAYAA-3′); HLRIR(T/S)K, P4 (5′-CATTTTWGTATTWGNWSNAA-3′); P1 and P2 (see above). Specific bands amplified after nested PCR were directly cloned and sequenced.

Isolation and reconstitution of cytochrome c oxidase. Cytochrome c oxidase was isolated either from mitochondria of bovine heart, liver and kidney using the non-ionic detergents Triton X-114 and Triton X-100, as described by Kadenbach et al. [28], or from mitochondrial particles of bovine heart as described by Yoshikawa et al. [29, 30]. The enzyme was reconstituted

into liposomes by hydrophobic adsorption of cholate to Amberlite XAD-2 [31], followed by dialysis. Purified asolectin (L-α-phosphatidylcholine, type II-s from soybean, Sigma) was sonicated to clarity in 1.5% sodium cholate, 100 mM K-Hepes, pH 7.4 at 40 mg/ml as previously described [17]. After addition of 3 μM cytochrome c oxidase and 5 mM (ATP + ADP) at variable ratios, or of other indicated nucleotides, the detergent was removed by adsorption to purified Amberlite XAD-2 from Sigma (50 mg/ml), via gentle shaking for 22 h at 4 °C. The liposomal suspension was then dialysed for 4 h against 200 volumes of 10 mM K-Hepes, pH 7.2, 27 mM KCl, 73 mM sucrose, and overnight against 200 volumes of 1 mM K-Hepes, pH 7.2, 30 mM KCl, 79 mM sucrose.

Measurement of H^+/e^- ratios. The H^+/e^- stoichiometry was measured by the reductant-pulse method as described before [14]. Into a thermostated (25 °C) open vessel, stirred mechanically from the top, proteoliposomes were suspended to a final concentration of 0.2 μM heme aa_3 in 1 mM K-Hepes, pH 7.0, 100 mM choline chloride, 5 mM KCl and 2 μg/ml valinomycin. The pH was measured with a microcombination pH electrode (U 402-M3 from Mettler Toledo) connected to a Beckman Expandometric IV pH meter. The H^+/e^- stoichiometry was determined from the initial pH decrease after addition of 6.4 μM ferrocytochrome c (8 enzyme turnover). The redox-linked pH changes elicited by pulses of ferrocytochrome c were calibrated with small aliquots of a standard solution of 10 mM HCl. The alkalinization due to the formation of water from O_2 was measured in the presence of 3 μM CCCP (carbonylcyanide m-chlorophenylhydrazone). For calculation of H^+/e^- ratios, the peak of acidification after addition of ferrocytochrome c was used [see ref. 14]. By extrapolation to zero time, as done in many previous publications, higher H^+/e^- ratios would be obtained.

Monoclonal antibodies reacting with subunits VIaH + VIc, but not with subunit VIaL [15] were prepared as described before [32]. IgG1 was purified from cell culture supernatants by chromatography on Protein A-Sepharose Fast Flow 4 (Pharmacia) in a 5-ml column and elution with 0.1 M sodium citrate, pH 4.5. The neutralized eluate (Tris base) was concentrated by centrifugation in Centricon 30 tubes (Amicon). The titer of the purified antibody was determined by ELISA titra-

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  1  ACACTGAGTT CAGCTGAGGA CAGACTGAAG GACAAACAAG CAGAGAAGAG
 51  GAGAAAATTA AATGAGTAAC GGTTTCGCCA GAAAAATAAG AAGAAAATAA

                                          M  S  L   S  A  L
101  AACATATTAG ATATTACCTG CCTGCAGAAC AATGTCTCTG TCTGCCTTGG

      A  A  R   R  V  F  A   A  A  S   H  S  S   H  E  G  G
151  CCGCTCGTCG TGTGTTTGCC GCTGCGTCGC ATTCAAGCCA TGAGGGAGGA

        A  R  T   W  K  I   L  S  F  V   L  A  A   P  G  V
201  GCGAGGACCT GGAAGATACT GTCCTTCGTG TTGGCCGCTC CTGGTGTCAC
                                                  P1
     T  V  C  M   I  N  A   Y  M  K   G  Q  A  H   S  H  E
251  CGTCTGCATG ATCAACGCCT ACATGAAGGG ACAGGCTCAC TCTCATGAAC

      P  P  E   F  V  P  Y   P  H  L   R  I  R   T  K  K  F
301  CGCCGGAGTT TGTGCCTTAT CCTCACCTGC GTATCCGAAC CAAGAAATTC

        P  W  G   D  G  N   H  S  L  F   H  N  P   H  T  N
351  CCTTGGGGAG ACGGAAACCA CTCTCTGTTC CACAACCCTC ACACCAACCC
           P2
     P  L  P  D   G  F  E   S  S  H   H  -
401  TCTGCCCGAT GGCTTCGAGA GCTCCCATCA CTGAGGAGAC CAGTCACATT
                                 P3
451  TCTTTAGCTG CACCTTTCTA AACTCCAGTC TGCAGTGTAC TTGGCTTAAT
           P2
501  TATACTGACC AAAGCAGAGG CGAAGCAGCA TCGCCATCAA GCGGTGCACT
551  CAGACAGGAA TAAAACCACT TTATGCTGTA TTAAAAAAAA AAAAAAAAAA
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Figure 1. cDNA sequence of cytochrome c oxidase subunit VIa from tuna heart, liver and the heater organ. The precursor sequence of the deduced amino acid sequence is shown in bold. The polyadenylation signal is underlined. The primers (P1–P4) are indicated with their 5′-3′ direction.

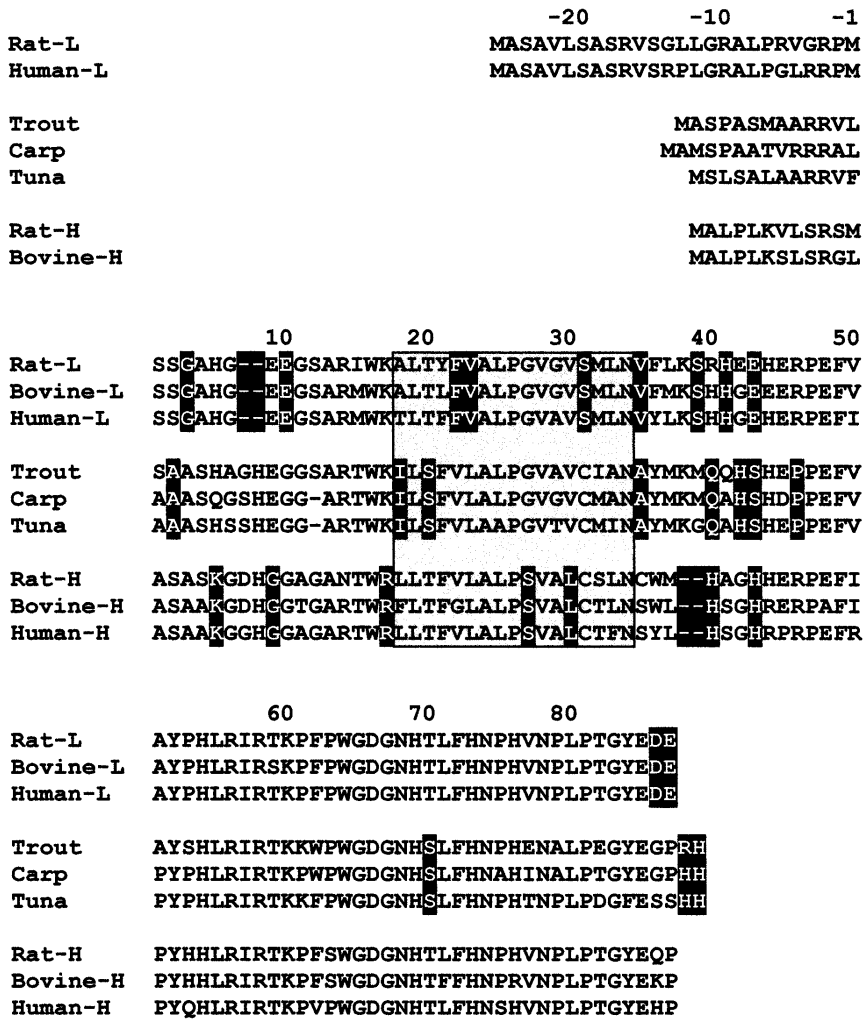

Figure 2. Comparison of the amino acid sequences of cytochrome c oxidase subunit VIa from tuna (this paper), carp and trout [22] and with the liver (L) and heart (H) isoforms of subunit VIa from rat, bovine and human [33]. Those amino acids of the fish-type, liver-type or heart-type of subunit VIa which are specific for this particular type are underlayed in black. The transmembraneous regions of the subunits are boxed.

tions [32]. Incubations of the purified monoclonal antibodies with the enzyme were performed either during reconstitution overnight (antibodies reacting with all accessible groups) or after reconstitution for 6 h at 4 °C (antibodies reacting only with extraliposomal groups).

Results

cDNA of tuna subunit VIa. PCR amplifications by 3′-RACE, followed by 5′-RACE, and cloning of the cDNAs for cytochrome c oxidase subunit VIa from liver, heart, skeletal muscle and the heater organ of tuna revealed various clones containing one and the same cDNA as shown in figure 1. Screening for subunit VIa isoforms in the four tuna tissues was performed by 3′-RACE-PCR, including nested PCR, with four different degenerated primers deduced from regions of subunit VIa which are homologous in all studied tissues and species (see Materials and methods). Under no conditions could bands containing sequences coding for proteins which were homologous to subunit VIa be amplified but different from the above cDNA for tuna subunit VIa. The same screening procedure, however, was successfully applied for the identification of a new isoform for another cytochrome c oxidase subunit (M. Hüttemann and B. Kadenbach, unpublished results). The deduced amino acid sequence of the mature protein is highly identical to that of subunit VIa from carp and trout, but shows low identity to subunit VIaH from

human, bovine and rat, as well as to subunit VIaL from these mammals (fig. 2). The fish-type subunit VIa is characterised by two additional C-terminal amino acids. Of particular interest is the deletion of two amino acids at different positions in subunits VIaH and VIaL, but not in the fish subunit. The deletion occurs either at the cytosolic domain (VIaL) or at the matrix domain (VIaH) of the transmembraneous subunit VIa [34]. Interestingly, the 12–14 amino acids of the fish precursor sequence correspond to the 12-amino-acid precursor of subunit VIaH, in contrast to the 26 amino acids of subunit VIaL. A phylogenetic tree of subunit VIa is presented in figure 3, based on sequence comparisons of the mature proteins. The figure extends previous phylogenetic trees of subunit VIa, based on sequences from mammalian species and yeast [33, 35]. From this most parsimonious phylogram, the separate evolution of three different types of subunit VIa in animals is suggested: the fish type, the mammalian skeletal muscle type (VIaH) and the mammalian non-skeletal muscle type (VIaL).

Variable H^+/e^- ratios of the heart enzyme. To elucidate the physiological function of tissue-specific isoforms of mammalian subunit VIa, we measured the H^+/e^- ratio of the reconstituted enzymes from bovine heart, liver and kidney. A previous publication described the decrease in the H^+/e^- stoichiometry of reconstituted cytochrome c oxidase of bovine heart from 1.0 to 0.5 by high intraliposomal ATP/ADP ratios [14]. This decrease in the H^+/e^- ratio is specific for ATP, because no nucleotide effect was obtained with increasing intraliposomal GTP/GDP ratios (not shown). With application of the non-hydrolysable ATP analog, PNP-AMP, and the GTP analog, PNP-GMP, almost the same H^+/e^- ratios were measured as obtained with 100% ATP and GTP, respectively (not shown). ATP interacts with the enzyme as a free anion, not complexed with Mg^{2+}, since the same H^+/e^- ratio was measured with the bovine heart enzyme after reconstitution either in the presence of 2 mM $MgCl_2$ or 2 mM EDTA (not shown).

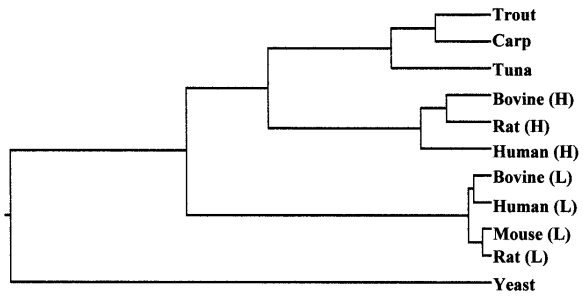

Figure 3. Most parsimonious phylogram describing the evolutionary relationship among cytochrome c oxidase subunits VIa. The comparisons were done with mature amino acid sequences using the Meg Align ©1993–97 program with the Jotun Hein algorithm [36]. The evolution of three different types of subunit VIa in animals is revealed.

Table 1. Comparison of the H^+/e^- ratios of different reconstituted cytochrome c oxidases from bovine heart, liver and kidney. The enzymes were isolated with Triton X-100 [28] or cholate only [29, 30], reconstituted without nucleotides, and the H^+/e^- ratios were determined as described in Materials and methods.

Enzyme	Isolation method	H^+/e^- ratio	Average H^+/e^- ratio
Heart	Triton X-100	0.66	
Heart	Triton X-100	0.85	
Heart	Triton X-100	0.75	0.75
Heart	Triton X-100	0.76	
Heart	Triton X-100	0.72	
Heart	cholate	0.93	
Heart	cholate	0.98	
Heart	cholate	0.66	0.85
Heart	cholate	0.85	
Heart	cholate	0.84	
Liver	Triton X-100	0.34	
Liver	Triton X-100	0.41	0.37
Liver	Triton X-100	0.37	
Kidney	Triton X-100	0.43	
Kidney	Triton X-100	0.36	0.39

ATP interacts with the matrix domain of subunit VIaH, as was demonstrated previously by reconstituting the bovine heart enzyme in the presence of a monoclonal antibody against subunits VIaH + VIc, which resulted in no decrease in the H^+/e^- ratio at high ATP/ADP ratios [14]. The monoclonal antibody does not react with the liver isoform of subunit VIa (VIaL) but does react with subunit VIc [15], due to an evolutionary relationship between subunits VIa and VIc [32]. The H^+/e^- ratio of the reconstituted enzyme from bovine liver was not influenced by the monoclonal antibody, indicating that the antibody effect on the heart enzyme is not due to interaction with subunit VIc (not shown). The antibody reacts with subunit VIa from the matrix side, since only when added during reconstitution, not after formation of the proteoliposomes, did the antibody prevent the decrease in the H^+/e^- ratio by high intraliposomal ATP/ADP ratios (not shown).

H^+/e^- ratio of the liver enzyme. In contrast to the isozyme from heart, the H^+/e^- ratio of reconstituted cytochrome c oxidase from bovine liver and kidney, containing subunit VIaL, is not influenced by high intraliposomal ATP/ADP ratios, as shown in figure 4. The measured H^+/e^- ratios of the liver and kidney enzyme, however, are in the same low range as mea-

sured with the heart enzyme at high intraliposomal ATP/ADP ratios. Table 1 presents the H^+/e^- ratios of various proteoliposome preparations of the three enzymes. When isolated by using Triton X-100, lower H^+/e^- ratios are usually obtained compared to those of enzymes isolated by using only cholate. But the H^+/e^- ratios of the heart enzyme preparations are always above 0.5, whereas those from liver and kidney are always below 0.5. The purity of the three enzyme preparations was the same, based on SDS-PAGE subunit patterns and on the heme a/protein ratios [28]. Therefore we postulate that the presence of subunit VIaL in the non-skeletal muscle isozyme reduces the H^+/e^- stoichiometry permanently from 1.0 to 0.5, independently of the matrix ATP/ADP ratio.

Discussion

In a previous study on cytochrome c oxidases from tuna heart and liver, the occurrence of isoforms for subunits Va, VIc, VIIb and VIII, but not for subunit VIa, was suggested [37]. The amino acid sequence of subunit VIa of tuna, deduced from identical cDNA sequences from liver, heart, skeletal muscle and the thermogenic organ, revealed high identity with that of carp and trout (fig. 2). In contrast, there are large differences between subunits VIa from fish and mammals. These data suggest that subunit VIa from tuna, like that of carp and trout, may not be involved in the suggested mechanism of thermogenesis of mammals, involving a decrease in the H^+/e^- ratio of cytochrome c oxidase at high ATP/ADP ratios via subunit VIaH [14, 18]. In fact, in tuna another mechanism of thermogenesis has been suggested, based on ATP-dependent cycling of Ca^{2+} ions across the sarcoplasmic reticulum of muscles and the thermogenic organ [25].

Cytochrome c oxidase from bacteria [38] and from eucaryotes is generally believed to pump protons at a H^+/e^- stoichiometry of 1.0 [39]. In contrast, the enzyme from mammalian heart, containing subunit VIaH, pumps protons at variable H^+/e^- ratios, depending on the intramitochondrial ATP/ADP ratio [14]. With an increasing ATP/ADP ratio, the H^+/e^- stoichiometry decreases from 1.0 to 0.5, with half-maximal decrease at ATP/ADP = 100 (fig. 4), corresponding to the physiological intramitochondrial ATP/ADP ratio. In the perfused heart, free cytosolic ADP levels of 30–90 μM have been calculated from ^{31}P-NMR data, resulting in ATP/ADP ratios of 200–1000 [40]. Due to the electrogenic nature of the ATP/ADP carrier, the ATP/ADP ratios are five to ten times lower in the mitochondrial matrix than in the cytosol. Therefore, intramitochondrial ATP/ADP ratios of 20–100 would be expected, corresponding to the measured regulatory range of H^+/e^- ratios.

Reconstituted cytochrome c oxidases from bovine liver and kidney always exhibited H^+/e^- ratios below 0.5, independent of the intraliposomal ATP/ADP ratio. Previous studies with isolated rat liver mitochondria yielded H^+/e^- ratios of 0.8 [41] and 0.6–0.7 [42]. For a long time, however, the H^+/e^- stoichiometry in cytochrome c oxidase was a matter of discussion [see references in refs 43, 44], for mainly methological reasons [45]. In our case, the H^+/e^- ratios were measured under identical conditions, with enzyme preparations from heart, liver and kidney of comparable purity, based on subunit composition and heme a/protein ratios. In our calculations we did not extrapolate the acidification peaks to zero time [see ref. 14], as done in most previous studies. Had we done so, we would have obtained higher H^+/e^- ratios for the liver-type and heart isozyme. Here we only point out that the heart enzyme at low ATP/ADP ratios reveals H^+/e^- ratios twice those of the liver-type isozymes.

A decrease of the H^+/e^- ratio from 1.0 to 0.5 would alone increase heat production by only 10%, since the total $H^+/2e^-$ ratio within the mitochondrial respiratory chain would be decreased from only 10 [46] to 9. The decrease in H^+/e^- ratio of the bovine heart enzyme, however, is accompanied by increased values of free energy, $-\Delta G^{o\prime}$, and decreased respiratory control ratios [18], resulting in increased rates of resting respiration and thus heat production. Correspondingly, the

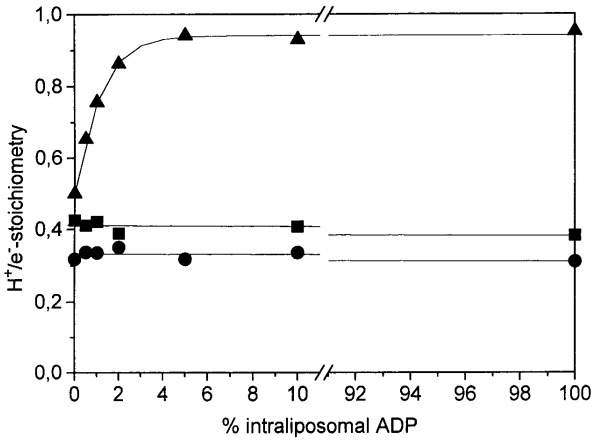

Figure 4. Intraliposomal nucleotides affect the H^+/e^- stoichiometry of the reconstituted enzyme from bovine heart, but not from bovine liver or kidney. Isolated cytochrome c oxidase from bovine heart (triangles), kidney (squares) and liver (circles) was reconstituted in the presence of the indicated percentage of ADP, where [ADP] + [ATP] = 5 mM, followed by dialysis. The pH was recorded during addition of 6.4 μM ferrocytochrome c to the proteoliposomes (0.2 μM heme aa_3) as described in Materials and methods.

respiratory control ratios (RCR) of the reconstituted enzymes from liver and kidney were usually lower (RCR = 3–4) than those of the heart enzyme at low ATP/ADP ratios (RCR = 5–9; data not shown). This could reflect intrinsic properties of the isozyme containing subunit VIaL, corresponding to the increased enzymatic activity of isolated cytochrome c oxidase from bovine liver compared to bovine heart [47, 48]. The control of cell respiration in vivo by cytochrome c oxidase was recently demonstrated by metabolic control analysis with cultivated cells [49, 50].

The molecular mechanism decreasing the H^+/e^- ratio in cytochrome c oxidase is not known. From the crystal structure of the bovine heart enzyme, two channels for proton pumping (A and B) and one channel for proton uptake for water formation (C) have been suggested [34] (see fig. 5). Two of these channels (A or D channel, and C or K channel) have also been identified in the enzyme from *Paracoccus denitrificans* [51], and postulated to solely contribute to proton pumping in the bacterial enzyme [52]. By comparison of high-resolution structures of the oxidized and reduced bovine heart enzyme, a large movement of Asp51 of subunit I, located at the cytosolic outlet of channel B, was found and suggested to contribute to proton pumping in the bovine heart enzyme [53]. In contrast, channel B was suggested not to be involved in proton pumping in the bacterial enzyme [54], although, except for a T→I exchange, all amino acids of this channel are highly conserved. Asp51, however, is not conserved in plant and bacterial enzymes.

As shown in figure 5, the C-terminal part of subunit VIaH is located close to the cytosolic outlet of the D channel (A) of the bovine heart enzyme. The decrease in H^+/e^- stoichiometry of the heart enzyme is apparently due to exchange of bound ADP by ATP at the matrix domain of subunit VIaH. This ADP (or ATP)-binding site was previously proposed [15] and verified in the crystal structure [34]. It may be absent in subunit VIaL, because only six high-affinity binding sites for ATP were measured by equilibrium dialysis in the bovine liver enzyme but seven in the bovine heart enzyme [55]. The C-terminal part of the three isoforms of subunit VIa are markedly different. Subunit VIaL contains two acidic amino acids at the end, which are neutral or basic in the other isoforms, and only the fish subunit contains two additional amino acids (fig. 2). Eucaryotic cytochrome c oxidase could principally pump protons either only through channel A with a stoichiometry of $H^+/e^- = 1.0$, or through channels A and B, each with a $H^+/e^- = 0.5$. Binding of ATP to the matrix domain of subunit VIaH could modify the interaction of its C-terminal domain with the outlet of channel A (D channel), thus inhibiting proton pumping through this channel. The lower H^+/e^- ratio could be due to either

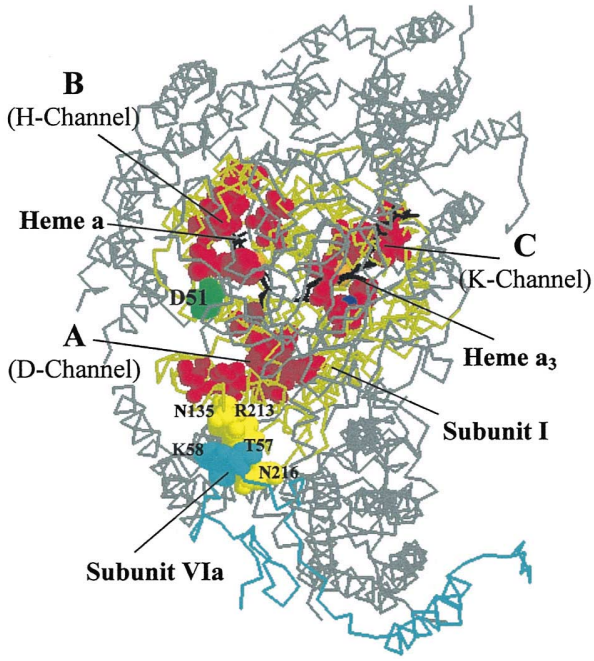

Figure 5. Crystal structure of one monomer of cytochrome c oxidase from bovine heart [34]. The crystallographic data were obtained from Protein Data Brookhaven (COX.pdb) and were processed by the RasMol 2.6 program. Shown is the cytosolic view on the membrane plane. Marked in colour are subunit I (yellow) and subunit VIa (cyan); all other subunits are indicated in grey. In red (half space filling) are marked those amino acids of subunit I which were suggested by Tsukihara et al. [34] to form the networks A and B, including D51 [53], for proton pumping and C for the uptake of protons for water formation. Heme a (close to channel B) and heme a_3 (within channel C) are coloured black. Cu_A is indicated in brown on the right of D51, Cu_B in blue. Amino acids of the outlet of channel A, which are in close contact to subunit VIa, are depicted in half space filling (yellow), the corresponding amino acids of subunit VIa in cyan. The distances between amino acids of subunit I to T57 of subunit VIa are: N216, 2.79 Å; N135, 3.11 Å; R213, 5.34 Å, and to K58 of subunit VIa are: R213, 3.48 Å; D144, 6.05 Å.

a decreased pumping stoichiometry through channel A from 1 to 0.5, or to closing channel A and proton pumping through only channel B ($H^+/e^- = 0.5$). Another possibility could be a decrease in the H^+/e^- ratio via interaction of the cytosolic tail of subunit VIaH with subunit III (not shown in fig. 5), which was assumed by Mather and Rottenberg [56] to constitute an exit channel for pumped protons of channel D. Subunit VIaL is suggested to permanently decrease proton pumping efficiency.

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