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Regulation of the H^+/e^- stoichiometry of cytochrome *c* oxidase from bovine heart by intramitochondrial ATP/ADP ratios

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Abstract **This paper describes the effect of intramitochondrial ATP/ADP ratios on the H^+/e^- stoichiometry of reconstituted cytochrome *c* oxidase (COX) from bovine heart. At 100% intraliposomal ATP the H^+/e^- stoichiometry of the reconstituted enzyme is decreased to half of the value measured below 98% intraliposomal ATP (above 2% ADP), while it remains constant up to 100% ADP. The decrease is obtained with different COX preparations, independent of the absolute value of the H^+/e^- stoichiometry. Decrease of H^+/e^- stoichiometry is prevented by preincubation of the enzyme with a tissue-specific monoclonal antibody to subunit VIa-H (heart-type). Tissue-specific regulation of the efficiency of energy transduction in COX of muscle mitochondria could have a physiological function in maintaining the body temperature at rest or sleep, i.e. at low ATP expenditure.**

Key words: Proton translocation; Cytochrome *c* oxidase; ATP; ADP; H^+/e^- stoichiometry; Tissue-specific regulation

1. Introduction

Cytochrome *c* oxidase (COX), the terminal enzyme of the mitochondrial and of various bacterial respiratory chains, transfers electrons from cytochrome *c* to oxygen, coupled with the vectorial uptake of protons for both, the formation of water ('chemical protons'), and for their translocation across the mitochondrial or bacterial membrane ('pumped protons'). Recently the crystal structure of COX from *Paracoccus denitrificans* [1] and from bovine heart [2–4] have been published at 2.8 Å resolution. These structures corroborated the known subunit compositions of the two enzymes; i.e. 4 subunits in the *Paracoccus* enzyme, and 13 in COX of bovine heart (3 mitochondrial coded and 10 nuclear coded subunits). Until recently, however, no function for the nuclear coded subunits of mammalian COX, not found in the bacterial enzyme, could be demonstrated. Indeed the functional properties of COX from bovine heart and from *Paracoccus* appeared indistinguishable [5,6].

In previous studies we have shown that intraliposomal ATP increases and ADP decreases the K_m for cytochrome *c* of reconstituted COX from bovine heart. However, the nucleotides had no effect on the reconstituted enzyme from *Paracoccus* [7]. In later studies intraliposomal ADP was shown to stimulate the activity of COX from bovine heart, but not from bovine liver. This stimulation could be inhibited by preincubation of the heart enzyme with a monoclonal antibody to the tissue-specific subunit VIa-H, indicating selective interaction of ADP with the N-terminal, matrix-oriented domain of subunit VIa-H [8]. In further studies a decrease of the respiratory control ratio at high intraliposomal ATP/ADP ratios was found with reconstituted COX from bovine heart, suggesting an inhibition of the H^+/e^- stoichiometry [9].

In the present study, the influence of various intraliposomal ATP/ADP ratios on proton translocation of reconstituted COX from bovine heart was measured directly with a pH-electrode using the 'reductant pulse' method. A decrease of H^+/e^- stoichiometry was found at high intraliposomal ATP/ADP ratios. This inhibition was not obtained with COX from bovine liver, and after preincubation of the enzyme with a monoclonal antibody to subunit VIa-H.

2. Materials and methods

COX was isolated from mitochondria of bovine heart and bovine liver, using the nonionic detergents Triton X-114 and Triton X-100, as described before [10]. The enzyme was reconstituted into liposomes by the hydrophobic adsorption method [11] 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 described [12]. After addition of 3 μM COX and 5 mM [ATP + ADP] at different ratios, 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.

The H^+/e^- stoichiometry was measured by the reductant-pulse method. Into a thermostated (20°C) open vessel, stirred mechanically from the top, was given 1.2 ml 1 mM K-HEPES, pH 7.0, 100 mM choline chloride and 5 mM KCl. After addition of 80 μl proteoliposomes (0.2 μM COX) and 1 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 7.7 nmol 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 water formation was measured in the presence of 3 μM CCCP (carbonylcyanide *m*-chlorophenylhydrazone).

Monoclonal antibodies reacting with subunit VIa-H and VIc, but not with subunit VIa-L [8] were prepared as described before [13]. IgG_1 was purified from cell culture supernatants by chromatography on protein A-Sepharose fast flow 4 (Pharmacia), essentially as described in [14], and concentrated by centrifugation in Centricon 30 tubes (Amicon). The titer of the purified antibody was determined by ELISA titrations [13]. COX from bovine heart or bovine liver (4 μM) was incubated in 100 mM K-HEPES, pH 7.4, with the equimolar amounts of the purified monoclonal antibody for 2 h at 4°C. Subsequent reconstitution of the enzymes in liposomes was performed as described above.

3. Results

COX from bovine heart was reconstituted into liposomes in the presence of variable portions of ATP and ADP but at a constant total concentration of [ATP + ADP] = 5 mM. Addi-

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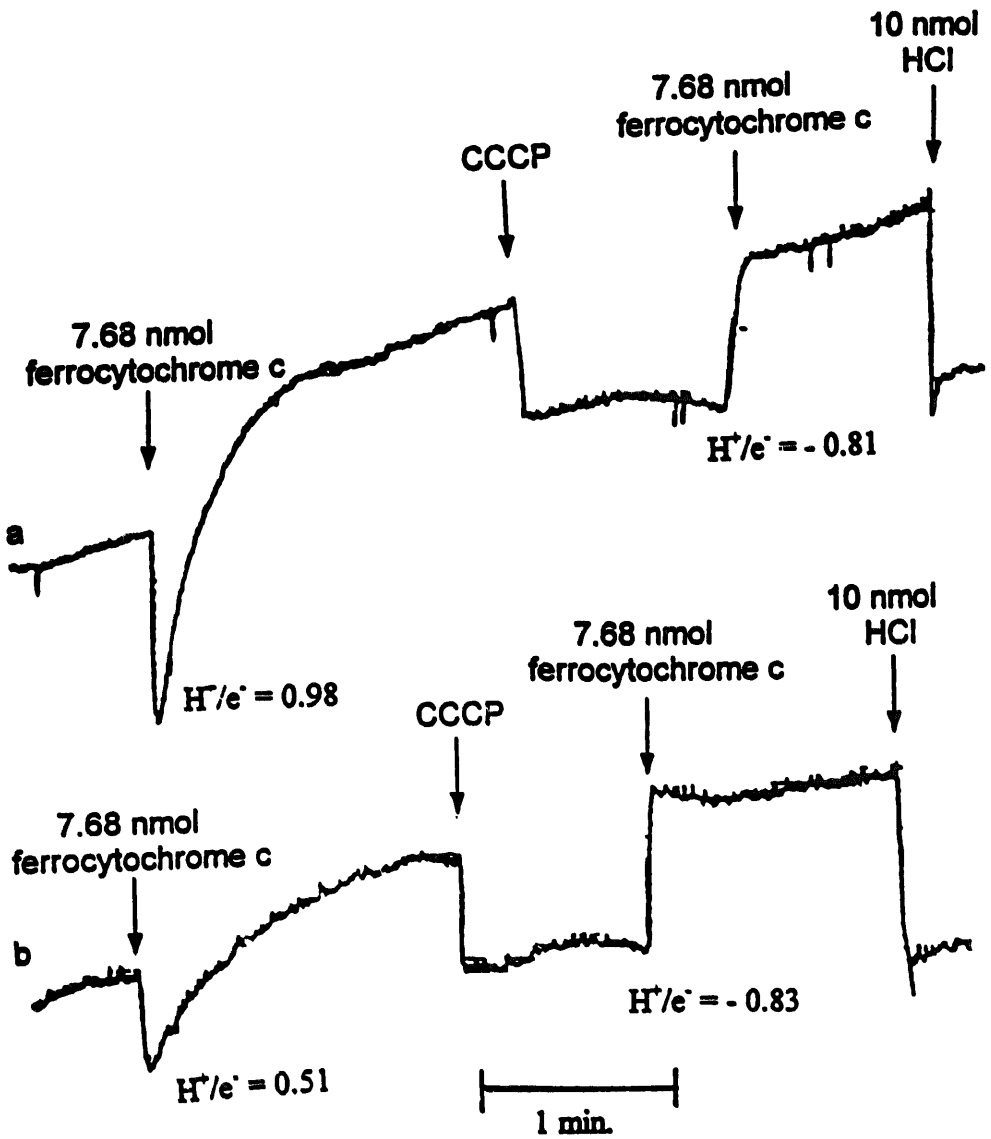

Fig. 1. Proton translocation of reconstituted COX from bovine heart, containing intraliposomal either 5 mM ADP (a) or 5 mM ATP (b). COX was reconstituted by the hydrophobic adsorption method in the presence of the indicated nucleotide. The extraliposomal nucleotides were removed by subsequent dialysis as described in section 2. The incubation system contained proteoliposomes (0.2 μM COX), 1 mM K-HEPES, pH 7.0, 100 mM choline chloride, 5 mM KCl and 1 μg/ml valinomycin. CCCP was added at a concentration of 3 μM where indicated.

tions of pulses of ferrocytochrome *c* to these proteoliposomal suspensions, containing the K^+ ionophore valinomycin, results in transient acidification, followed by alkalinization, as shown in Fig. 1. In the presence of the uncoupler CCCP no transient acidification is obtained. Instead, after addition of ferrocytochrome *c*, an immediate alkalinization is observed, because the uptake of protons from the intraliposomal space for the formation of water is immediately equilibrated between inner and outer compartment via the uncoupler. In Fig. 1a the proteoliposomes contained in the inner compartment 5 mM ADP; and in Fig. 1b, 5 mM ATP. With vesicles containing ATP the peak of acidification is about 50% smaller than with vesicles containing ADP. The calculated H^+/e^- stoichiometries are 0.98 in the presence of ADP and 0.51 in the presence of ATP. After addition of CCCP the H^+/e^- stoichiometries are almost the same and amount to −0.81 and −0.83 for the two vesicle preparations, respectively. Addition of 4 mM $MgCl_2$ during reconstitution did not change the result (not shown). Thus, it appears that the free nucleotides, not the magnesium complexes, are bound to the enzyme.

The H^+/e^- stoichiometries of vesicles containing increasing amounts of ADP, is presented in Fig. 2 for different preparations of COX from bovine heart. At 5 mM ATP (0% ADP) in the intraliposomal space of the proteoliposomes, the H^+/e^- stoichiometry is about 50% lower than that obtained at concentrations below 98% ATP (above 2% ADP). Between 2% and 100% ADP within the vesicles the H^+/e^- stoichiometry remains constant. Also, vesicles containing no intraliposomal nucleotides showed the same H^+/e^- stoichiometry than those with 5 mM intraliposomal ADP (not shown). The decrease of the H^+/e^- stoichiometry at high intraliposomal ATP/ADP ratios is independent of the maximal value of the H^+/e^- stoichiometry. This follows from the data obtained with 3 differ-

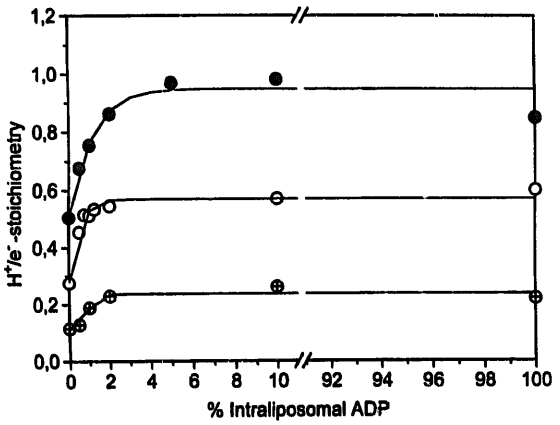

Fig. 2. Decrease of the H^+/e^- stoichiometry of reconstituted COX from bovine heart at high intraliposomal ATP/ADP ratios. COX was reconstituted by the hydrophobic adsorption method as described in section 2 in the presence of the indicated percentages of ADP, but at constant total concentration of [ATP + ADP] = 5 mM. The subsequent dialysis removed all extraliposomal nucleotides. Presented are data obtained with proteoliposomes prepared with three different COX preparations (closed, open and crossed circles, respectively). The values of the curve with closed circles represent the average of two independent measurements.

ent COX preparations (Fig. 2, closed, open and crossed circles), where the H^+/e^- stoichiometry varies between 0.2 and 0.9. The maximal H^+/e^- stoichiometry was found to vary between different COX preparations, depending on the concentration of ammonium sulfate at which the enzyme was precipitated [10].

In Fig. 3 the effect of preincubation of COX from bovine heart with a monoclonal antibody to subunit VIa-H (heart-type), which does not react with subunit VIa-L (liver-type) of COX from bovine liver [8], is shown. The H^+/e^- stoichiometry of proteoliposomes, containing the antibody-treated enzyme, is not decreased at high intraliposomal ATP concentrations. Pretreatment of COX from bovine heart with an unspecific IgG from bovine (Sigma) did not prevent the inhibition of H^+/e^- stoichiometry at high intraliposomal ATP/ADP ratios (not shown). The same results as shown in Fig. 3, were also obtained with different preparations of COX from bovine heart, pretreated with the monoclonal antibody to subunit VIa-H.

4. Discussion

This paper describes regulation of proton translocation of reconstituted COX from bovine heart by intraliposomal ATP/ADP ratios. High ATP/ADP ratios decrease the H^+/e^- stoichiometry to maximally half of the value measured at 98% or lower ATP portions. The results corroborate previous studies on the inhibition of the respiratory control ratio (RCR) of reconstituted COX from bovine heart at high intraliposomal ATP/ADP ratios [9]. In these studies the RCR_{Val} (related to the respiration in the presence of valinomycin), which was shown by Wilson and Prochaska [15] to represent a measure of the H^+/e^- stoichiometry, was stronger inhibited than the RCR (related to the respiration in the absence of uncoupler).

Two observations of this study suggest that the decrease of H^+/e^- stoichiometry at high intraliposomal (i.e. intramitochondrial) ATP/ADP ratios has physiological significance. (1) The regulation is prevented by preincubation of COX from bovine heart with a monoclonal antibody to subunit VIa-H (heart isoform), which does not react with subunit VIa-L (liver isoform) of COX from bovine liver [8]. The two enzymes differ in isoforms for subunits VIa, VIIa and VIII [10]. It is assumed that both, ATP and ADP bind to the N-terminal (matrix-oriented) domain of subunit VIa-H, as previously suggested [8]. (2) Regulation of H^+/e^- stoichiometry in COX appears to be tissue-specific. With reconstituted COX from bovine liver no decrease of H^+/e^- stoichiometry was obtained at high intraliposomal ATP/ADP ratios. In this experiment the H^+/e^- stoichiometry, however, was rather low ($H^+/e^- = 0.3$) (data not shown).

The binding site for ATP (or ADP), postulated to be located at the N-terminal domain of subunit VIa-H [8], has in fact recently been localized in the crystal structure of the bovine heart enzyme [3]. The function of 6 further ATP binding sites in COX of bovine heart and 6 ATP binding sites in COX of bovine liver [18] remains to be established. These include an ATP binding site localized at the C-terminal domain of subunit VIa of COX from yeast, bovine heart and bovine liver [19].

The fact that inhibition of H^+/e^- stoichiometry occurs only above 98% of intraliposomal ATP, or below 2% of ADP, suggests that ADP is bound at the same site as ATP but with higher affinity. In contrast to bound ADP, which does not influence the H^+/e^- stoichiometry, bound ATP is assumed to change the conformation of COX, accompanied by a decrease of the H^+/e^- stoichiometry. This mechanism could only be of physiological significance if the free intramitochondrial ADP concentration could be lower than 2% from total nucleotides. Measurement of total ADP levels from whole tissue extracts overestimates the free ADP concentrations by approximately 20-fold due to the large number of intracellular sites which sequester ADP [16]. In fact the free intracellular ADP level is low and was determined to 0.05 μmol/g wet weight (about 50 μM) for mouse liver [17]. The free ADP level within the mitochondria is unknown. Although the intramitochondrial ATP/ADP ratio is lower than the cytosolic

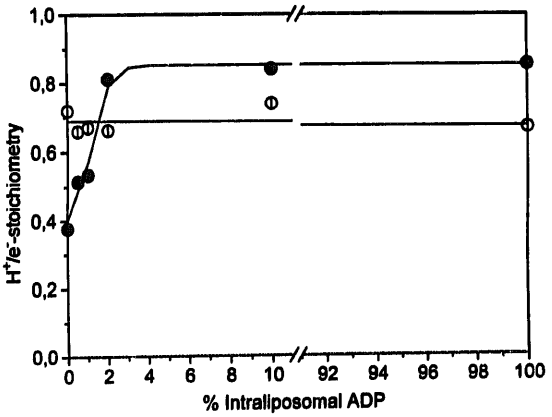

Fig. 3. Influence of preincubation of COX from bovine heart with a monoclonal antibody to subunit VIa-H on the inhibition of H^+/e^- stoichiometry of the reconstituted enzyme by high intraliposomal ATP/ADP ratios. Closed circles = proteoliposomes reconstituted with untreated COX in the presence of the indicated amounts of nucleotides ([ATP/ADP] = 5 mM); open circles = proteoliposomes reconstituted with COX, pretreated with the monoclonal antibody to subunit VIa-H.

one [20], due to the mitochondrial membrane potential and the electrogenic nature of the ADP/ATP carrier, the low total cellular ADP level suggests also high ATP/ADP ratios in the mitochondrial matrix, since [ATP+ADP] concentration is about 15 mM in rat liver mitochondria [20].

Decrease of the H^+/e^- stoichiometry in skeletal muscle mitochondria at high matrix ATP/ADP ratios, i.e. at rest or during sleep, will result in increased thermogenesis if the rate of electron transfer remains constant. This mechanism was suggested to participate in maintenance of body temperature in mammalian organisms, containing about 70% of muscle tissue [9,12].

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