Electrogenic proton exchange between cytochrome a_3 active center and M-aqueous phase

Evidence for cytochrome a_3-associated input proton well

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The rate of cyanide binding with the oxidized cytochrome-*c* oxidase in proteoliposomes is controlled by ionization of a protein group with p$K \sim 6.7$, the ligand reacting with the protonated enzyme only [(1983) Bioorg. Chem. (USSR) 9, 216–227]. As shown here, the kinetics of cyanide binding depends on the pH inside the proteoliposomes. The reaction rate is affected by the electrical potential difference across the proteoliposome membranes as if the a_3-linked ionizable group exchanged H^+ with the proteoliposome interior electrogenically. The data corroborate a hypothesis on the existence of a proton well communicating cytochrome oxidase O_2-reducing center with the M-aqueous phase.

Cytochrome-c oxidase *Proteoliposome* *Proton well* *Cyanide binding* *Heme-linked ionizable group* *Proton pump*

1. INTRODUCTION

Mitochondrial cytochrome-*c* oxidase operates as a redox-driven proton pump [1,2], but little is known about the mechanism underlying the protonmotive function of the enzyme.

According to a model suggested in [3,4] and considered by other workers [5,6], cytochrome *c* oxidase has three proton wells communicating the redox-linked ionizable groups of the enzyme with the M- and C-aqueous phases (see the scheme in fig.3; for other possible mechanisms, see [7]). Two of these proton wells (I and II in fig.3) were tentatively assigned to cytochrome *a*, whereas cytochrome a_3 was suggested to have only an 'entrance' or 'input' well III providing for electrogenic protonation of reduced oxygen intermediates from the M-aqueous phase [3,8].

Earlier, evidence for the cytochrome *a* associated input proton well I was obtained [4,9,10]. Here we report on an active center-linked ionizable group of cytochrome a_3 which exchanges protons electrogenically with the M-aqueous phase.

2. METHODS

2.1. *Chemicals*

Cytochrome *c* type VI, asolectin (phosphatidylcholine type IIS), choline chloride, Good buffers, carbonyl cyanide *m*-chlorophenylhydrazone, cholic and deoxycholic acids were from Sigma. The bile acids were purified by recrystallisation from a charcoal-treated 70% ethanol solution as described in [11]. KCN and NaCN (Analar) were from BDH or Merck. Nigericin and monensin were from Calbiochem-Behring.

2.2. *Preparations*

Cytochrome-*c* oxidase was isolated and purified from beef heart mitochondria according to

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[12,13]. The enzyme was reconstituted into asolectin vesicles by the cholate dialysis method [14]. For preparation of the low-buffered K^+- or Na^+-loaded proteoliposomes, 160–200 mg asolectin were dispersed in 4 ml of the medium containing 0.1 N K^+ or Na^+ salt (chloride or sulfate), 5 mM Hepes-Tris, pH 7.5, 2% sodium cholate and 2 mM $MgSO_4$. The suspension was flushed with argon and sonicated with a Brownsonic-1510 disintegrator equipped with a microtip at approx. 80 mV output or with a UZDN-2T disintegrator operated at 22 kHz and 0.5 A until clarification of the mixture. 4–5 mg cytochrome oxidase protein were then added and the mixture was dialyzed for 4–5 h against 1 l of the same buffer containing no cholate and then overnight against the same cholate-free buffer adjusted to pH at which the experiment was to be carried out. Before the experiments, the proteoliposomes were passed or centrifuged [15] through a Sephadex G-25 column to remove external K^+ or Na^+. The K^+,Na^+-free proteoliposomes were prepared similarly but substituting K^+ or Na^+ salts for 0.1 M choline chloride and omitting the Sephadex column step.

2.3. *Assays*

The kinetics of cyanide binding with ferric cytochrome oxidase was monitored in an Aminco $DW2^a_{TM}$ spectrophotometer in a stirred 1 cm rectangular cell thermostatted at 27°C.

3. RESULTS

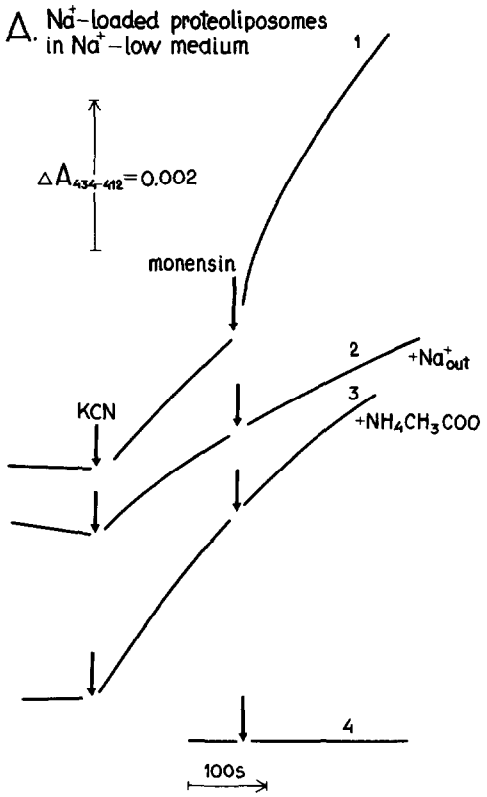
It is known that in pigeon heart [16] and rat liver [8] mitochondria, ferric cytochrome a_3 combination with HCN requires protonation of a heme-linked ionizable group with pK 6.6–7.0. More recently the phenomenon has been studied in considerable detail with the beef heart enzyme [17,18], and the kinetics of the ligand binding has proved to be a sensitive indicator of the cytochrome a_3 active center protonation [8,18].

Here, we attempted to find out whether it is pH inside or outside the cytochrome oxidase proteoliposomes that affects the cyanide binding kinetics. In the experiment shown in fig.1A, Na^+-loaded proteoliposomes with a low internal buffering capacity are suspended in an essentially K^+,Na^+-free strongly buffered medium. The reaction is initiated with KCN and monensin is added which exchanges internal Na^+ for external H^+ and produces acidification specifically inside the vesicles as verified by appropriate control experiments with 9-aminoacridine and pyranine as fluorescent pH indicators (not shown, see [19] and cf. [20]). It can be seen that under these conditions monensin greatly stimulates the reaction (trace 1). The effect of monensin is prevented by external NaCl (trace 2), and by CH_3COONH_4 which neutralizes internal acidification (trace 3).

The stimulation of the cyanide binding can also be observed with K^+-loaded proteoliposomes using nigericin instead of monensin (fig.1B). On the contrary, when K^+,Na^+-free proteoliposomes are suspended in a potassium-rich medium, nigericin which would now catalyze alkalinization of the proteoliposome interior impedes a_3^{3+} ligation by cyanide (fig.1C, trace 1). The effect is prevented by an uncoupler (fig.1C, trace 2) and is not observed in the absence of external K^+ (not shown). The inhibition of cyanide binding with a_3^{3+} in the K^+,Na^+-free proteoliposomes can also be imposed by monensin in the presence of 50 mM Na_2SO_4 outside the vesicles (not shown). In the absence of cyanide, monensin or nigericin do not bring about any significant changes of absorbance at the indicated wavelengths (fig.1A, trace 4 and C, trace 3).

These effects of monensin and nigericin on the kinetics of cyanide binding indicate that the ionizable group which controls heme a_3 reactivity towards the ligand responds to pH inside the vesicles.

It is noteworthy that with respect to cytochrome oxidase topography, the interior of the proteoliposomes in our experiments is equivalent to the matrix aqueous phase of mitochondria. First, about 80–85% of cytochrome oxidase molecules in our preparation of proteoliposomes proved to be in the mitochondrial orientation as probed by the ascorbate + cytochrome c reducibility [21], which is in agreement with [22]. Second, the experiments have been carried out in the presence of cytochrome c and at low concentrations of cyanide. Earlier we showed [17,18,23] that ferric cytochrome c greatly stimulates combination of HCN with a_3 lowering K_m of the reaction more than 100-fold. Thereafter, the experimental conditions were chosen in this work so as to allow for HCN binding at a measurable rate only with the



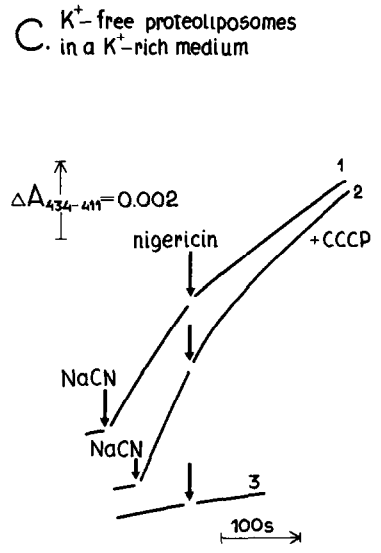


Fig.1. Effect of acidification and alkalinization of the proteoliposome interior on the kinetics of cyanide binding with ferric cytochrome oxidase. (A) Interior acidification. Cytochrome oxidase proteoliposomes loaded with 50 mM Na_2SO_4 ([aa_3] = 0.4 μM). The essentially Na^+-free basic medium containing 50 mM Hepes-Tris buffer, pH 8.3, 2 mM $MgSO_4$, 0.12 mM $K_3Fe(CN)_6$ and 2 μM cytochrome *c* is supplemented with 0.1 M choline chloride and also: (2) 0.1 M NaCl or (3) 20 mM CH_3COONH_4. Additions: KCN, 10 μM; monensin, 2 ng/ml. (B) Interior acidification. Proteoliposomes loaded with 0.1 M KCl ([aa_3] = 0.5 μM). The basic medium (as in A, but pH 7.5) is supplemented with 0.1 M choline chloride (1) or 0.1 M KCl (2). Additions: NaCN, 27 μM; nigericin, 0.13 μM. (C) Interior alkalinization. Proteoliposomes essentially free of internal K^+ and Na^+ ([aa_3] = 0.5 μM). The basic medium with pH 7.7 is supplemented with 0.1 M KCl and also (2) 3 μM carbonyl cyanide *m*-chlorophenylhydrazone. Additions: NaCN, 50 μM; nigericin, 0.13 μM.

cytochrome *c*-stimulated cytochrome oxidase molecules, i.e. with the mitochondrially oriented fraction of the enzyme. Thus we can conclude in accordance with [3,8] that the heme-linked ionizable group of cytochrome a_3 which controls the ligand binding is in a protonic contact with the M-aqueous phase. The question now arises as to whether this proton exchange is electrogenic.

An inhibition of HCN binding with ferric a_3 upon addition of ATP to pigeon heart mitochondria was observed originally by Wilson and Fairs [24] who attributed the effect to direct structural interaction of the ATPase and cytochrome oxidase complexes. In contrast, we suggested [3] and showed [8,18] that it is $\Delta\psi$ across the membrane of energized mitochondria that impedes the reaction

raising K_m for HCN, i.e. exerting the same effect as alkalinization. A model was proposed according to which the cytochrome a_3 active center is localized at the bottom of a proton well extending to the M-aqueous phase so that a transmembrane electric field of a normal sign (positive on the C-side) results in a deprotonation of the heme a_3 surroundings [3,8] (see fig.3).

A crucial prediction of the hypothesis was that $\Delta\psi$ of opposite polarity (negative on the C-side) should concentrate H^+ at the bottom of the a_3-associated proton well and, at pH above pK of the a_3-linked protolytic group, would stimulate HCN combination with cytochrome a_3. As shown in fig.2, this is indeed the case. When K^+-free proteoliposomes are suspended in 50 mM K_2SO_4 buffered at pH 8.3 the rate of HCN binding with heme a_3^{3+} is greatly stimulated by valinomycin (trace 1). The effect of valinomycin is not observed in the absence of K^+ gradient (trace 2) and is largely prevented by the uncoupler (trace 3). At pH 6.8, which is close to pK of the a_3-linked ionizable group [16–18], the stimulating effect of the K^+ diffusion potential becomes much less pronounced (trace 4). Some unidentified increment of absorbance at 434 minus 412 nm was sometimes observed upon addition of valinomycin to proteoliposomes in the absence of cyanide (in the presence of a K^+ gradient only, see [18]); however, these unspecific absorption changes (the largest response among a dozen control recordings is given in fig.2 by trace 5) obviously cannot account for the effect observed in the case of cyanide binding kinetics (trace 1).

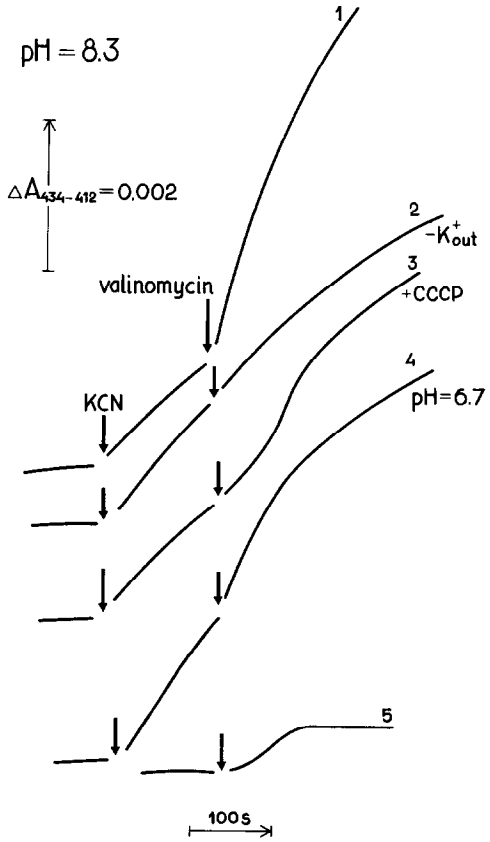


Fig.2. Stimulation of cyanide binding with ferric cytochrome oxidase by $\Delta\psi$ of opposite polarity imposed across the proteoliposome membrane. K^+-free proteoliposomes with a strongly buffered (50 mM Hepes-Tris) interior ($[aa_3]$ = 0.4 μM). The basic medium is supplemented with: (1,4,5) 50 mM K_2SO_4; (2) 0.1 M choline chloride; (3) 50 mM K_2SO_4 + 1 μM carbonyl cyanide *m*-chlorophenylhydrazone. 1–3,5, pH 8.3; 4, pH 6.8. Additions: KCN, 20 μM (1–3) or 10 μM (4); valinomycin, 1 μM.

4. DISCUSSION

The strategy of a search for a proton well in an energy-transducing membrane enzyme, as used here, comprises 3 sequential steps: (i) to reveal an active center-linked acid-base group(s); (ii) to determine with which side of the coupling membrane the group is in a protonic contact; (iii) to look for the effect of transmembrane electric field on the protonic equilibrium between the group and the corresponding aqueous phase.

The presence of an ionizable group with pK 6.6–7.0 controlling cyanide binding to ferric cytochrome a_3 was described [8,16–18]. The identity of this group remains to be established. It was suggested to be a heme iron-coordinated axial histidine [16], a distal (non-coordinated) histidine [3], or an H_2O/OH^- molecule at the 6th axial position of heme a_3 iron in the 'pulsed' conformation of cytochrome oxidase [8]. Notably, K_m of cyanide combination with a_3^{3+} which is the pH-dependent parameter of the reaction [8,16–18] may correspond to K_d of the ligand binding at a site other than heme a_3 iron [25], possibly $Cu_{a_3}^{2+}$ [26]. Therefore, the ionizable group in question may be, actually, Cu_{a_3}- rather than Fe_{a_3}-linked.

The present data together with our earlier results

on intact mitochondria [8,18] indicate that this group exchanges protons with the M-aqueous phase and that this exchange is electrogenic. Thus, this group is likely to be localized at the bottom of the cytochrome a_3-associated input proton well as illustrated by fig.3.

Accordingly, oxygen protonation in the cytochrome oxidase reaction is associated with H^+ uptake from the M-phase [1,27,28]. However, in the absence of adequately time-resolved (i.e. sub-millisecond) pH kinetics measurements, this H^+ uptake from the M-phase could not be assigned to any particular step of the multiphase reaction mechanism and, in fact, might be coupled to the enzyme turnover in a somewhat complex way.

The significance of the present data is that they reveal electrogenic H^+ exchange between the heme a_3/Cu_{a3} surroundings and M-aqueous phase in a well defined state of the enzyme. Conceivably, this H^+ exchange could be a partial reaction of the catalytic cycle: as mentioned earlier [3], the ionizable group at the bottom of the a_3-associated proton well could serve as an immediate H^+ donor to the Fe_{a3}/Cu_{a3}-bound reduced oxygen intermediates.

Finally, we hope that the general approach used here may help to identify proton wells in membrane energy transducers other than cytochrome oxidase.

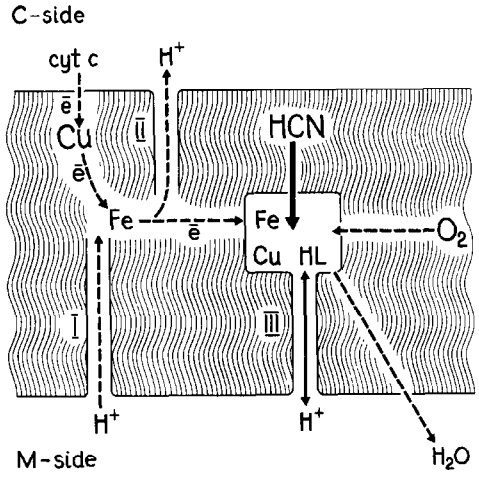


Fig.3. A proposed mechanism of the $\Delta\psi$ effect on cyanide binding with cytochrome a_3. A general diagram of the cytochrome oxidase energy-transducing mechanism with 3 proton wells in adapted form [3,4]. The reactions which are not operative under the conditions of the present experiments with the oxidized enzyme are given by dashed lines. Cyanide can bind to the oxidized a_3 active center only when the ionizable group L is protonated. Just as one possibility, protonation of L could weaken its coordination to Cu^{2+}_{a3} and allow for its displacement by cyanide. Protonation of L is suggested to occur by virtue of electrogenic H^+ exchange with the M-aqueous phase via an input proton well III and is sensitive to electric potential difference across the membrane. $\Delta\psi$ of the physiological sign (positive on the C-side) will deprotonate LH and decelerate cyanide binding (as shown in [8,18]), whereas $\Delta\psi$ of the opposite sign will protonate L and accelerate the reaction (present results).

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