Vectorial nature of redox Bohr effects in bovine heart cytochrome *c* oxidase

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Abstract The vectorial nature of redox Bohr effects (redox-linked pK shifts) in cytochrome *c* oxidase from bovine heart incorporated in liposomes has been analyzed. The Bohr effects linked to oxido-reduction of heme *a* and Cu_B display membrane vectorial asymmetry. This provides evidence for involvement of redox Bohr effects in the proton pump of the oxidase.

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Key words: Cytochrome *c* oxidase; Redox Bohr effect; Liposome; Proton pump

1. Introduction

Redox Bohr effect in cytochromes, i.e. thermodynamic linkage between oxido-reduction of the metal centers and the pK's of protolytic groups in the enzymes, result in pH dependence of the midpoint redox potentials [1] and in proton transfer associated to oxido-reduction of the metal centers [2]. The H^+/e^- linkage in cytochromes is likely to arise from modification of the coordination bonds of metal centers associated with change in their valence state. The linkage can involve pK shifts and exchange of axial ligands [3,4]. The H^+/e^- linkage can also involve porphyrin substituents [5,6] and conformational propagation of primary effects over long distances in the protein [7], as in the oxygen Bohr effect of hemoglobin [8].

Recent analysis of H^+ transfer associated to redox transitions of the metal centers in the soluble cytochrome *c* oxidase from bovine heart, in the unliganded, CN- and CO-liganded state has identified four groups undergoing reversible redox-linked pK shifts [9]. Two protolytic groups resulted in being linked to redox transitions of heme a_3, one group to redox transitions of Cu_B, the fourth group to oxido-reduction of heme *a*.

Redox Bohr effects are likely to play a role in the exchange of protons between aqueous phases and protolytic redox reactions in the membrane environments, i.e. protonation of the intermediates of oxygen-reduction to H_2O in heme copper oxidases [10]. Mechanisms have also been suggested in which redox Bohr effects are conceived to participate in proton pumping by the cytochrome systems of the respiratory chain (vectorial Bohr mechanism) [7,11,12]. Considerable attention has been paid by different groups to protolytic events associated with the catalytic process in cytochrome *c* oxidase [12–16]. A 'histidine cycle' has been put forward to explain coupling in cytochrome *c* oxidase between oxido-reductions at the binuclear heme a_3-Cu_B center and proton pumping [3,4]. Furthermore, distinct possible proton pathways have been detected in the crystal structure of the oxidase for the transfer of the scalar protons (to be consumed in the reduction of oxygen to H_2O) and the pumped protons respectively [4,17,18].

It appears of critical relevance to these issues to identify the four protolytic groups whose pK's are linked to redox transitions of the metals in the oxidase [9] as well as to clarify their vectorial organization in the membrane. We have now analyzed the vectorial nature of the redox Bohr effects in the cytochrome *c* oxidase from bovine heart reconstituted in liposomes (COV). The proton transfer resulting from the Bohr effects linked to heme *a* and to Cu_B shows membrane vectorial asymmetry, i.e. protons are taken up, upon reduction of the metals, from the inner space and released in the outer space upon their oxidation. These findings provide direct evidence supporting a vectorial Bohr mechanism in the proton pump of cytochrome *c* oxidase.

2. Materials and methods

2.1. Enzyme preparation and reconstitution in liposomes

Cytochrome *c* oxidase was purified from beef heart mitochondria as described in [19]. The nmoles of heme a+a_3/mg protein were in both cases about 10 and SDS-PAGE analysis revealed the complete set of 13 subunits [20]. The activity of the enzyme preparation measured polarographically was greater than 300 TN/s. Reconstitution of cytochrome *c* oxidase in phospholipid vesicles (COV) was performed by the cholate dialysis method as described in [21] with the following differences: (i) the aa_3/phospholipid ratio was doubled (final concentration of cytochrome *c* oxidase was 6 μM); (ii) in the last dialysis medium 1 mM K-HEPES was omitted. These changes did not affect the respiratory control ratio, which was even higher than in the 'standard' procedure (never below 15, when measured polarographically [22]) or the right-side-out orientation of the oxidase molecule in the liposomal membrane (never below 80% [23]).

2.2. Measurement of pH and redox changes

Simultaneous recordings of absorbance and pH changes were carried out by a diode array spectrophotometer (in the multiwavelength kinetic analysis mode) and a fast response combined pH electrode respectively [2], with accuracies of 1×10^{-4} absorbance and 10^{-3} pH unit (overall response time < 1 s). Redox transitions of heme a+a_3 and cytochrome *c* were monitored at 605–630 nm ($\Delta\varepsilon = 13.5$ mM^{-1}) [24] and 550–540 nm ($\Delta\varepsilon = 19.1$ mM^{-1}) [25] respectively. In the CO-liganded state heme *a* oxidation was followed at 604–630 nm ($\Delta\varepsilon = 21.9$ mM^{-1}), and the generation of the mixed valence state at 590–630 nm ($\Delta\varepsilon = 10.0$ mM^{-1}) [26]. Ferricyanide solutions were assayed at 420–500 nm ($\Delta\varepsilon = 1.00$ mM^{-1}).

3. Results

Fig. 1 shows the analysis of the redox Bohr effects in the CO-liganded oxidase reconstituted in liposomes at pH 7.4. Cytochrome *c* oxidase was fully reduced by succinate by means of a trace of broken mitochondria and cytochrome *c*

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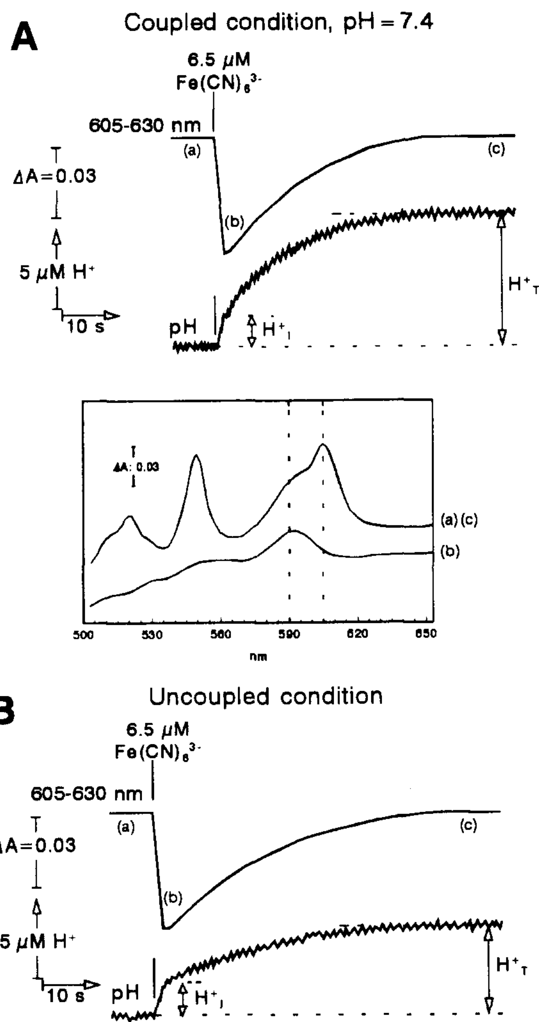

Fig. 1. Analysis of the sidedness of the H^+ transfer associated with redox transitions of metal centers in the CO-liganded cytochrome *c* oxidase vesicles. 2.5 μM COV were suspended in 0.15 M KCl and supplemented with 2.5 μM cytochrome *c*, 1 μg/ml valinomycin and 0.1 mg/ml frozen thawed beef heart mitochondria, pH 7.4 (panel A). The suspension was gently bubbled with CO for 2 min and then covered with a layer of mineral oil to prevent further gas exchange. Addition of 3 mM succinate produced anaerobiosis and full reduction of cytochrome *c* oxidase in 10–15 min, where indicated anaerobic 6.5 μM ferricyanide was added and heme *a* absorbance and pH changes monitored simoultaneously as described in Section 2. The panel in the middle shows the difference spectra collected after addition of succinate and upon re-reduction of the metal centers (a) and (c) respectively, characteristic of the fully reduced CO-liganded aa_3, and after addition of ferricyanide (b), characteristic of the mixed valence CO-liganded aa_3. After the redox cycle, 3 μM CCCP was added and the addition of ferricyanide repeated (panel B). Control experiments where mitochondria were omitted or blocked with antimycin A showed that the addition of the same amount of ferricyanide did not result in any change in pH or absorbance. H^+_I and H^+_T refer to the initial acidification following the rapid oxidation of the metal centers, upon addition of ferricyanide, and to the total amount of H^+ released at the end of the redox cycle respectively (see text and Table 1).

in the presence of CO. The panel in Fig. 1A shows the characteristic spectrum generated by CO binding to the reduced enzyme (spectrum a). Addition of a small amount of ferricyanide, substechiometric with the redox metals in the oxidase, oxidized heme *a* and Cu_A (oxidation of Cu_B and heme a_3 was blocked by CO) generating the mixed valence oxidase (spectrum b).

The oxidation of heme *a* and Cu_A was accompanied by synchronous release of H^+ which continued, as expected, during the re-reduction of the metal centers by succinate (Fig.

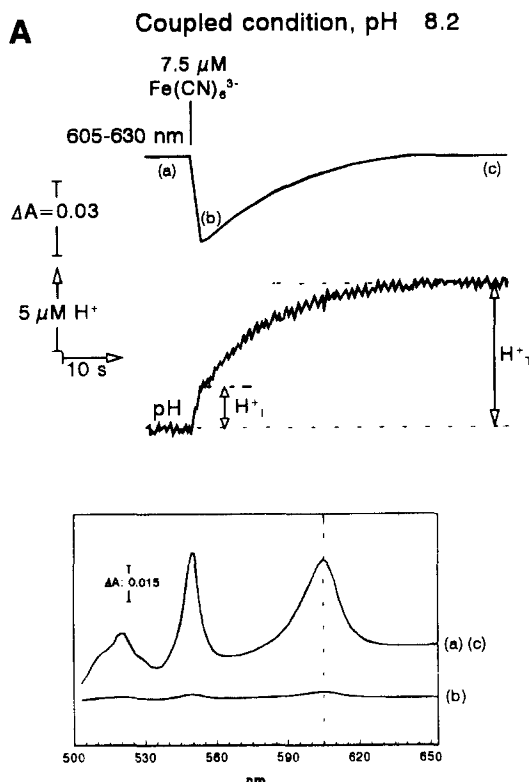

Fig. 2. Analysis of the sidedness of the H^+ transfer associated with redox transitions of metal centers in the unliganded cytochrome *c* oxidase vesicles. 1.5 μM COV were suspended in the same medium as in Fig. 1 supplemented with 2.0 μM cytochrome *c*, 1.0 μg valinomycin and 0.1 mg/ml of mitochondria, pH 8.2. The suspension was bubbled with N_2 and the experiment performed as described in the legend of Fig. 1. Panels A and B refer to the 'redox cycle' performed in the absence and in the presence of 3 μm CCCP respectively. The panel in the middle shows the difference spectra collected during the course of the experiment at the indicated points (a), (b) and (c). See text and Tables 1 and 2 for further details.

Table 1
Analysis of scalar H^+ transfer linked to redox transitions of the metal centers in cytochrom *c* oxidase vesicles

Exp. conditions	$Fe(CN)_6^{3-}$ added (μM)	Heme *a* oxidized (μM)	H_T^+ (μM)	H_I^+ (μM)	$H_T^+/Fe(CN)_6^{3-}$	ΔH^+/COX	H_I^+/COX
CO-liganded, pH 7.4							
Coupled	6.50	2.33	8.50	2.05	1.31	0.86	0.88
Uncoupled	6.50	2.33	5.78	2.10	0.89		0.90
		Heme a+a_3 oxidized (μM)					
Unliganded, pH 8.2							
Coupled	7.50	2.54	8.62	2.81	1.15	0.88	2.21
Uncoupled	7.50	2.54	7.35	3.05	0.98		2.40

For experimental conditions with the CO-liganded and unliganded cytochrome *c* oxidase see Figs. 1 and 2 respectively.
H_I^+ refers to the initial H^+ release associated to the rapid oxidation of metal centers elicited upon addition of ferricyanide.
H_T^+ refers to the total amount of H^+ released following the oxidation-reduction cycle.
Internal measurements showed that the total amount of redox carriers oxidized was equivalent to the amount of ferrycianide added. Oxidation of cytochrome *c* and hemes *a* and a_3 was directly estimated by absorbance changes. The equivalents of Cu_A undergoing oxido-reduction were assumed to be equal to those measured for heme *a* in the CO-liganded and unliganded conditions. The equivalents of Cu_B undergoing oxidoreduction were assumed to be equal to those measured for heme *a* in the unliganded conditions.
ΔH^+/COX refers, under coupled conditions, to the ratio between the extra H^+ release with respect to the amount of ferricyanide added and the amount of cytochrome *c* oxidase undergoing oxido-reduction.
H_I^+/COX refers to the ratio between the initial H^+ release associated to the rapid oxidation of metal centers and the amount of cytochrome *c* oxidase. See text for further details.

1A). The overall reduction of added ferricyanide by succinate should have resulted in the 1 to 1 stoicheiometric release of H^+ in the external medium. An extra-acidification was, however, observed which was significantly larger than the amount of ferricyanide added and amounted to 0.86 H^+ per oxidase molecule (see Table 1). There is clearly an additional source of H^+ which appears to be associated with the transient oxidation of heme *a*, the oxidation of cytochrome *c* and Cu_A is irrelevant in this respect as they are pH independent [27,28]. It is thus evident that Bohr protons associated with the oxidation of heme *a* (amounting to 0.86 ± 0.07 H^+/COX at pH 7.4, see Fig. 4B of [9]), are released in the external aqueous phase (Table 1A). If the redox Bohr protons were then taken up by the oxidase from the same external aqueous phase upon re-reduction of heme *a* by succinate, no net excess of H^+ release, with respect to the ferricyanide added, should have been left when heme *a* was fully re-reduced, as it was instead observed. The remaining extra-acidification, which was equal to the rapid H^+ release associated with heme *a* oxidation (Table 1), shows that the Bohr protons associated with re-reduction of heme *a* are taken up from the inner aqueous phase. This was confirmed by the observation that in the presence of CCCP, which equilibrates the inner and outer pH changes, the same initial rapid acidification was observed upon oxidation of heme *a* by ferricyanide, as in the coupled system, but at the completion of the re-reduction of heme *a* no extra-acidification, with respect to the ferricyanide added, was observed (Fig. 1B, Table 1).

The same measurements were then carried out with the reconstituted unliganded oxidase. An experiment at pH 8.2 is shown in Fig. 2 (see also Table 1). At this pH there is no contribution of the Bohr effect of Cu_B (see Fig. 5 in [8]). The metal centers in cytochrome *c* oxidase were fully reduced by succinate by means of the trace of broken mitochondria and cytochrome *c* which brought the suspension to anaerobiosis. The oxidation of the metal centers of the oxidase by a sub-

Table 2
Statistical analysis of the sidedness of H^+ transfer linked to redox transitions of the metal centers in reconstituted cytochrome *c* oxidase vesicles

Expt. conditions	*n*	H_T^+/Ferricyanide added	ΔH^+/COX	H_I^+/COX
CO-liganded, pH 7.4				
Coupled	8	1.25 ± 0.02	0.86 ± 0.07	0.90 ± 0.10
Uncoupled	8	0.90 ± 0.05		
Unliganded, pH 8.2				
Coupled	12	1.21 ± 0.02	0.92 ± 0.08	2.17 ± 0.09
Uncoupled	12	1.02 ± 0.01		
Unliganded, pH 6.7				
Coupled	9	1.15 ± 0.01	1.17 ± 0.08	1.60 ± 0.10
Uncoupled	9	0.96 ± 0.03		

Results: mean ± S.E.M.
For experimental conditions with the CO-liganded and unliganded cytochrome *c* oxidase see Figs. 1 and 2 respectively.
H_T^+/*Ferricyanide*: total amount of H^+ released following the oxidation-reduction cycle elicited by the addition of ferricyanide, divided by the amount of ferricyanide added.
Internal measurements showed that the total amount of redox carriers oxidized was equivalent to the amount of ferrycianide added.
ΔH^+/COX refers under coupled conditions to the ratio between the extra H^+ release with respect to the amount of ferricyanide added and the amount of cytochrome *c* oxidase undergoing oxido-reduction; the difference between the mean values estimated in the unliganded conditions at pH 6.7 and 8.2 was statistically significant ($P < 0.05$).
H_I^+/COX refers to the ratio between the initial H^+ release associated to the rapid oxidation of metal centers and the amount of cytochrome *c* oxidase.
n is the number of the experiments performed.

stechiometric amount of ferricyanide resulted at pH 8.2 in the release of 2.2 H^+/COX (Table 1) which was very close to the H^+ release corresponding to Bohr effects associated with oxidation of heme *a* and a_3 (see Fig. 4A of [9]). Also in the unliganded oxidase, when re-reduction by succinate of the metal centers of the oxidase was completed an extra-acidification, with respect to the ferricyanide added, was left. This extra-acidification which amounted to 0.88 H^+/COX was equivalent to that observed with the CO-liganded oxidase and practically corresponded to the Bohr protons associated with heme *a*. It can thus be concluded that both in the unliganded and CO-liganded oxidase the redox Bohr effect linked to heme *a* displays membrane vectorial asymmetry, i.e. protons are taken up by the enzyme from the inner aqueous phase upon reduction of heme *a* and released in the external aqueous phase upon its oxidation. The redox Bohr protons linked to heme a_3 appear, on the other hand, to be released in, upon oxidation, and taken up, upon reduction, from the external aqueous space.

The membrane vectoriality of the redox Bohr effect linked to Cu_B was clarified by a statistical analysis of the redox Bohr effects in the unliganded COV at pH 6.7 where the redox Bohr effect for Cu_B reaches the peak of 0.3 H^+/COX. The data obtained at this pH indicate that the Bohr protons linked to oxidation of Cu_B are released, as those of heme *a* and heme a_3, in the external space (see Table 2), but are taken up, as those linked to heme *a*, from the inner space upon its re-reduction. This is clearly shown by the fact that the extra-acidification observed at the completion of the ferricyanide-induced redox cycle (ΔH^+/COX) was at pH 6.7 larger than at pH 8.2, 1.17 ± 0.08 and 0.92 ± 0.08 ($P < 0.05$) respectively, and the difference between these two values practically corresponded to the Bohr protons linked to Cu_B (see Fig. 5 of [9] and Table 2). It can be noted that in the unliganded enzyme the initial acidification (H_I^+/COX) measured upon ferricyanide addition was larger than the extra-acidification (ΔH^+/COX) measured at the end of the redox cycle. The difference between the initial and final extent of acidification practically corresponds to the Bohr protons associated to heme a_3 which are released in, upon oxidation, and taken up, upon re-reduction, from the external aqueous phase.

4. Discussion

What presented shows that the proton transfer resulting from the redox Bohr effects linked to heme *a* and Cu_B in the bovine heart cytochrome oxidase display membrane vectorial asymmetry, i.e. the protons are taken up from the inner aqueous space, upon reduction, and released in the external space, upon oxidation of the metals. This direction of the proton uptake and release is just what is expected from a vectorial Bohr mechanism in which redox-linked cooperative events are conceived to be extended over the transmembrane span of the enzyme so as to result in proton uptake from the inner and their release in the external aqueous phase [7].

The two groups whose pK's change upon oxido-reduction of heme a_3 [9] exchange protons only with the external aqueous phase. This would exclude a role of these two groups in proton pumping. The group linked to heme *a*, whose pK changes upon reduction from 6 to around 9 and which can transfer up to 0.9 H^+ per e^- [9] should provide the major contribution to the proton pump (cf. [29,30]). The group linked to Cu_B undergoes, upon reduction, only a small increase in its pK, from 6.4 to 7.0, and consequently does not transfer more than 0.3 H^+ per e^- [9].

The histidine cycle proposed for the proton pump of cytochrome *c* oxidase is based on redox-linked binding changes at Cu_B of an invariant histidine ligand which cycles between Cu_B-bound imidazolate (Im^-) and free imidazolium ($ImHH^+$) [3,4]. The group identified here as linked to Cu_B with pk_{ox} and pk_{red} values around 6 and 7 respectively, could be a histidine in the imidazole/imidazolium state (pK 6.5) or a H_2O/OH^- molecule [31]. This seems, in principle, to be incompatible with the proposed binding to Cu_B of a histidine in the imidazole/imidazolate state (pK around 14) as postulated in the histidine cycle. The pK shift of the group linked to Cu_B is also too small to represent an energetically relevant step in the pump.

The nature of the group linked to heme *a* remains to be determined. Since this center does not bind O_2, O_2-reduction intermediates or other ligands, the group in question is likely to be an amino acid residue in subunit I. Coupling of proton translocation to electron transfer via heme *a* could take place, at least for a part of its route, along the hydrogen bond network seen in the crystal structure of the bovine oxidase to be contributed by residues of transmembrane helices XI and XII of subunit I [18]. It has been proposed that this putative proton pathway can be controlled by redox changes of heme *a*, as the OH group of the farnesyl chain of the porphyrin is hydrogen bonded to *Ser382* in the proton pathway [18].

The critical role in proton pumping of the vectorial Bohr effect linked to heme *a* seems to be supported by the correspondence of the pH dependence curves of this effect with that of the H^+/e^- ratio for proton pumping in purified cytochrome *c* oxidase vesicles [22]. The crystal structure of the bovine oxidase shows, in addition to the residue networks providing electron transfer from Cu_A to heme *a* and from the latter to heme a_3-Cu_B, another residue network which could mediate direct electron transfer from Cu_A to heme a_3-Cu_B [18]. Enhanced contribution of this latter pathway, as seems to occur at high electron pressure or when the proton-motive electron transfer via heme *a* is depressed by the back pressure of aerobic $\Delta\mu H^+$, has been proposed to explain the decoupling of the proton pump observed under these conditions [32].

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