Redox-linked protolytic reactions in soluble cytochrome-*c* oxidase from beef-heart mitochondria: redox Bohr effects

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Abstract

A study is presented of co-operative redox-linked protolytic reactions (redox Bohr effects) in soluble cytochrome-*c* oxidase purified from bovine-heart mitochondria. Bohr effects were analyzed by direct measurement, with accurate spectrophotometric and potentiometric methods, of H^+ uptake and release by the oxidase associated with reduction and oxidation of hemes a and a_3, Cu_A and Cu_B in the unliganded and in the CN- or CO-liganded enzyme. The results show that there are in the bovine oxidase four protolytic groups undergoing reversible pK shifts upon oxido-reduction of the electron transfer metals. Two groups with pK_{ox} and pK_{red} values around 7 and > 12 respectively appear to be linked to redox transitions of heme a_3. One group with pK_{ox} and pK_{red} around 6 and 7 is apparently linked to Cu_B, a fourth one with pK_{ox} and pK_{red} of 6 and 9 appears to be linked to heme a. The possible nature of the amino acids involved in the redox Bohr effects and their role in H^+ translocation is discussed.

1. Introduction

Cytochrome-*c* oxidases from eukaryotes and prokaryotes have four redox centers [1–3]. A Cu_A binuclear center, which titrates as a one electron redox entity [4], serves as the entry port in the oxidase for the electrons delivered by cytochrome *c* [5]. Heme a transfers electrons from Cu_A to the heme a_3-Cu_B binuclear center, where dioxygen is reduced

to H_2O and electron transfer seems to be directly coupled to proton pumping [5]. The midpoint redox potentials of heme a and a_3 decrease as the pH of the medium is raised within the physiological pH range [6–9]. The redox potential of Cu_A appears to be pH-independent [7,9,10], while that of Cu_B has been reported to show similar pH dependence to heme a [11]. The pH dependence of hemes a and a_3, like that of the *b* cytochromes [12], indicates a linkage between the valence state of the heme-iron and protolytic events in the enzymes (redox Bohr effects) [13–17]. 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 could be confined locally and

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involve pK shifts of protolytic axial ligands, as in cytochrome c [18] or the oxidant Bohr effect in hemoglobin and myoglobin, which results from change in the ionization of a water molecule at the sixth coordination position in the heme iron [13]. The H^+/e^- linkage could also involve porphyrin substituents [19,20] and/or conformational propagation of primary effects over long distances in the protein, as in the oxygen Bohr effect of hemoglobin [13,21].

Redox Bohr effects may play a role in the exchange of protons between aqueous phases and protolytic redox reactions in membrane environments [16]. Mechanisms have also been suggested in which Bohr effects could participate in vectorial proton translocation by the cytochrome system [14–17,22]. Recently a 'histidine cycle' has been put forward to explain coupling in cytochrome-c oxidase between redox transitions at the binuclear Cu_B-heme a_3 center and proton pumping [23]. This model is based on redox-linked binding changes at Cu_B and pK shifts of an invariant histidine ligand [23,24] (cf. Ref. [25]).

Analysis of the pH dependence of the redox centers in the oxidase is made difficult by the complex co-operative behaviour they display [1,26], by the difficulty in spectrally discriminating the two hemes and by the spectral 'invisibility' of Cu_B [1]. We have now extended previous direct measurements [27–29] (see also Refs. [30–32]) of scalar proton transfer associated with oxido-reduction of the metal centers in unliganded, cyanide-liganded and CO-liganded cytochrome-c oxidase isolated from beef-heart mitochondria. Analysis of the pH dependence curves of the experimentally measured H^+/e^- coupling ratios in the unliganded and liganded enzyme allowed us to identify different pK shifts linked specifically to redox transition of the individual redox centers. In particular two pK shifts were found to be associated with heme a_3 and one each with heme a and Cu_B.
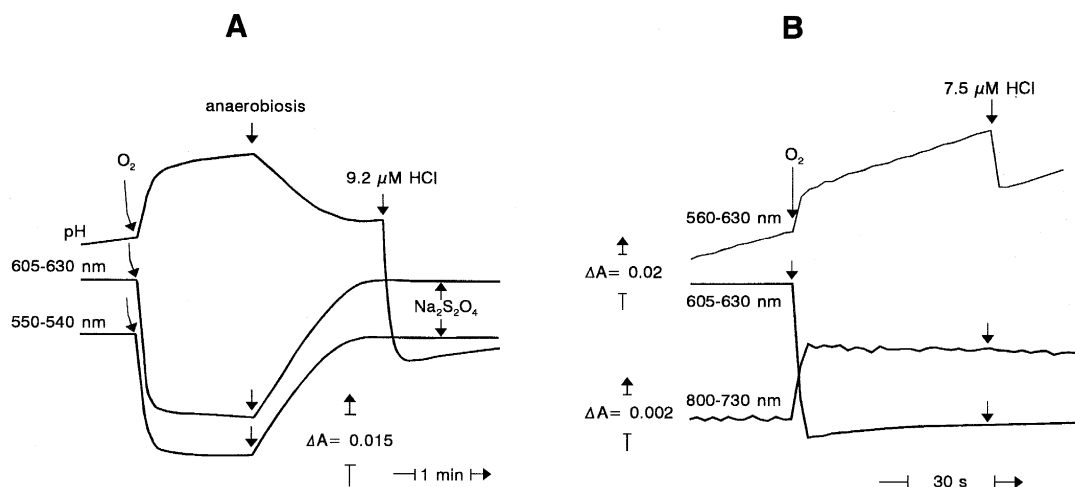

Fig. 1. Scalar H^+-transfer associated with oxido-reduction of metal centers in unliganded purified cytochrome-c oxidase. (A) 1.55 μM cytochrome-c oxidase was suspended in 0.25 M sucrose, 50 mM KCl, 0.5 mM EDTA supplemented with 2 μM cytochrome c, 0.1 mg protein/ml rat liver mitochondria (0.05 μM aa_3), 1 μg rotenone/ml, 1 μM CCCP, pH 7.2. After 5 min incubation under continuous stream of argon, the cuvette was stoppered with a gas-tight plug containing two holes, one fitted by the pH-electrode, the other by a microsyringe needle. After addition of 5 mM K-succinate anaerobiosis was reached in about 10 min. 2 mM malonate was then added and oxygenation was brought about by repetitive additions of oxygenated medium (5 μM dioxygen). Oxidoreduction of hemes $a + a_3$, cytochrome c and Cu_A (not shown) was monitored spectrophotometrically, pH changes were monitored potentiometrically (see under Section 2). (B); 3 μM cytochrome-c oxidase was suspended in 0.15 M KCl, 0.015% dodecyl maltoside supplemented with 10 mM glucose, 100 μg glucose oxidase/ml, 0.05 μM catalase, 5 mM ascorbate, 0.1 μM ruthenium hexamine and 50 μM phenol red, pH 7.2. The cuvette was stoppered with a rubber plug with no gas phase left and placed in a diode array spectrophotometer set in the multi-wavelength kinetic analysis mode. Anaerobiosis and full reduction of heme a was achieved in about 15 min. Spectra from 400 to 800 nm (recorded in 0.1 s) were collected every 3 s. Oxygen was admitted unplugging the cuvette under continuous stirring conditions. Absorbance and pH changes were monitored spectrophotometrically. For further experimental details see Section 2. For quantitative data see Table 1, columns A and B.

The possible role of these redox Bohr effects is discussed.

2. Materials and methods

2.1. Enzyme preparation

Cytochrome-c oxidase was purified from beef heart mitochondria either as described in Ref. [33] or in Ref. [34]. The nmol heme $a + a_3$/mg protein were in both cases about 10 and SDS-PAGE analysis revealed the complete set of 13 subunits [35]. The activity of the two enzyme preparations measured polarographically was greater than 300 TN/s.

2.2. Measurement of pH and redox changes

In a set of experiments (see legends to figures) simultaneous recordings of absorbance and pH changes were carried out by double-wavelength spec-

trophotometry and potentiometry respectively [16], with accuracies of $5 \cdot 10^{-4}$ absorbance and 10^{-3} pH unit (overall response time < 1 s). In another set of experiments pH changes were monitored, simultaneously with redox transitions, following absorbance changes of the pH indicator phenol red (absorbance changes at 560–630 nm) with a diode array spectrophotometer in the multi-wavelength kinetic analysis mode. Redox transitions of heme $a + a_3$ and cytochrome c were monitored at 605–630 nm ($\Delta\epsilon = 13.5$ mM^{-1}) [36] and 550–540 nm ($\Delta\epsilon = 19.1$ mM^{-1}) [37] respectively. Redox transitions of Cu$_A$ were monitored at 830–740 nm or 800–730 nm ($\Delta\epsilon = 1.0$ mM^{-1}) [38]. In the KCN-inhibited cytochrome-c oxidase redox changes of heme a were monitored using a $\Delta\epsilon$ at 605–630 nm of 21.6 mM^{-1} assuming a 80% contribution of heme a to the overall absorbance, at the indicated wavelength couple, in the unliganded state [39]. In the CO-liganded state heme a oxidation was followed at 604–630 nm ($\Delta\epsilon = 21.9$ mM^{-1}), and the generation of the mixed

Table 1
Analysis of scalar H$^+$ transfer linked to redox transitions of the metal centers in purified soluble cytochrome-c oxidase

	μM	Unliganded		CN-liganded			CO-liganded
		A	B	C	D	E	F
	COX used	1.55	3.00	8.00	2.20	1.50	1.20
i.	H$^+$ measured uptake and/or release	4.78	5.45	2.15	5.13	1.84	0.88
	cytochrome c	1.89	–		0.67	1.86	
	Cu$_A$ *	1.48	2.94	1.13	2.11	1.45	1.15
	heme a	1.52	2.98	3.26	2.15	1.48	1.15
	heme a$_3$	1.52	2.98	–	–	–	–
	Cu$_B$	1.48	2.94	3.08	2.11	1.45	–
ii.	Σe^-	7.89	11.84		7.04	6.24	
iii.	a. shortfall (ii-i)	3.11	6.39		1.91		
	b. shortfall (0.5 ii-i)					1.28	
	Bohr H$^+$ (iii)/COX	2.05	2.14		0.89	0.86	
	Bohr H$^+$ (i)/heme a			0.66			0.76

* It should be noted that Cu$_A$ binuclear center titrates as one electron redox entity [4].

For experimental details see Figs. 1–3. Columns A and B refer to the experiments shown in Fig. 1A,B; columns D and E to the experiments in Fig. 2A,B; column F to the experiment in Fig. 3. The shortfalls in H$^+$ transfer were obtained from the difference between the measured H$^+$ (consumed or released) and the scalar H$^+$ transfer expected from the equivalents of the metal centers undergoing oxido-reduction. These were: 1 H$^+$ consumed per metal oxidized in the reduction of O$_2$ to H$_2$O (experiments A and B); 1 and 0.5 H$^+$ released per metal center reduced by succinate and ascorbate respectively (experiments D and E). In the experiments A,B,D,E the concentration of active COX was estimated from the average of its metal centers undergoing redox transition. In the experiment of column C, 8 μM cytochrome-c oxidase, incubated overnight with 2 mM KCN in 0.15 M Kcl at pH 7.2, was subjected to reduction-oxidation cycles by successive pulses of 10 μM ferrocytochrome c and 15 μM ferricyanide. Heme a and Cu$_A$ oxidoreductions were followed spectrophotometrically; the equivalents of Cu$_B$ undergoing oxidoreduction were considered to be equivalent to those measured for Cu$_A$ in experiments A, B, D and E or those estimated from the differences between the equivalents of ferrocytochrome c oxidized and heme a and Cu$_A$ reduced in experiment C.

valence state at 590–630 nm ($\Delta\epsilon = 10.0$ mM^{-1}) [36].

2.3. Data analysis

The pH dependence of the observed redox-linked H$^+$-transfer reactions, expressed as H$^+$/COX ratio, was best fitted with curves obtained using Eq. (1):

$$H^+/COX = \Sigma i |1/(1 + 10^{pH - pK_{ox}})$$
$$- 1/(1 + 10^{pH - pK_{red}})| \qquad (1)$$

which gives the theoretical pH dependence of the H$^+$/COX ratio for redox-Bohr effects attributable to protolytic group(s) with different pKs (pK_{ox} and pK_{red}) in the oxidised and reduced state.

3. Results

Redox Bohr effects in soluble, unliganded cytochrome-c oxidase were analysed by repetitive cy-cles of reduction and oxidation of the enzyme [16]. The oxidase solution supplemented with a stoicheio-metric concentration of cytochrome c and a small, catalytic amount of broken mitochondria was allowed to become anaerobic by succinate respiration in the presence of CCCP. This resulted in full reduction of the metal centers as revealed by spectrophotometric monitoring of hemes a + a$_3$ (see Fig. 1A) and Cu$_A$ (not shown). Addition of oxygen induced re-oxidation of all the added cytochrome c and of the reduced metal centers of the oxidase, which resulted in net H$^+$ uptake. Upon anaerobiosis re-reduction of the oxidase centers by succinate, which proceeded at a rate of 10^{-2} electrons $\cdot$ s^{-1} $\cdot$ aa$_3^{-1}$ (as compared to a turnover of the enzyme of 300), resulted in net H$^+$ release (Fig. 1A). The H$^+$ uptake and release were, however, smaller, by the same amount in each case, than the sum of the electron carriers undergoing oxido-reduction. This shortfall gives a direct measure of H$^+$ release and uptake from protolytic groups in
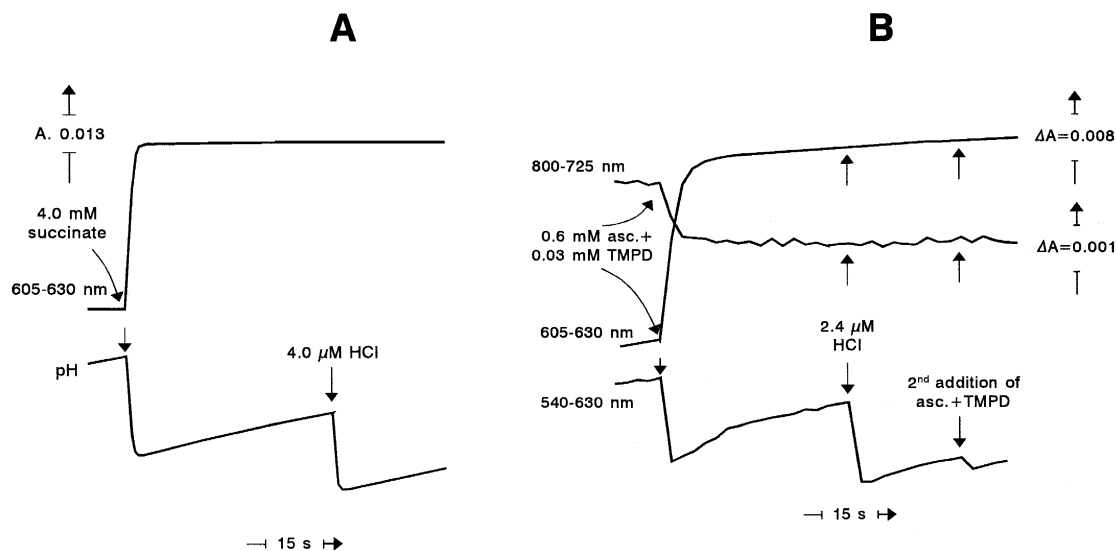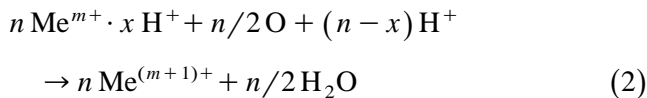

Fig. 2. Scalar H$^+$ transfer associated with reduction of metal centers in CN-liganded cytochrome-c oxidase. (A) 2.2 μM cytochrome-c oxidase was suspended in the medium described in the legend to Fig. 1A supplemented with 2 mM KCN, 0.7 μM cytochrome c and incubated at 4°C overnight. Heme a and cytochrome c reduction (not shown) and associated H$^+$ release elicited upon addition of succinate were simultaneously monitored spectrophotometrically and electrometrically respectively. (B) 1.5 μM cytochrome c oxidase was suspended in 0.15 M KCl, 0.015% dodecyl maltoside supplemented with 2 mM KCN, pH 7.2, and incubated at 4°C overnight. Just before measurements 50 μM phenol red and 1.86 μM ferricytochrome c were added. Absorbance and pH changes were monitored by diode array spectrophotometry as described in the legend to Fig. 1B. The reaction was started by addition of a freshly prepared mixture of 0.6 mM ascorbate plus 30 μM TMPD at pH 7.2. A second addition was made to verify that full reduction of heme a and Cu$_A$ was achieved and to quantify a small artifact caused by the ascorbate solution itself.

the oxidase associated with oxido-reduction of the metal centers (reaction 2) [16]:

$$n\,\mathrm{Me}^{m+}\cdot x\,\mathrm{H}^+ + n/2\,\mathrm{O} + (n-x)\,\mathrm{H}^+$$
$$\rightarrow n\,\mathrm{Me}^{(m+1)+} + n/2\,\mathrm{H_2O} \qquad (2)$$

The value of x for the overall Bohr effects at pH 7.0 was 2.05 H^+ per oxidase molecule undergoing oxido-reduction (Table 1, column A).

A similar experiment was carried out using ascorbate and a substoicheiometric amount of ruthenium hexamine (Ru II) as reductant of cytochrome-c oxidase (see Fig. 1B). The sample was supplemented with glucose/glucose oxidase/catalase to facilitate achievement of anaerobiosis. Once full reduction of hemes $a + a_3$, $\mathrm{Cu_A}$ (directly monitored) and (presumably) $\mathrm{Cu_B}$ was attained, $\mathrm{O_2}$ was admitted to the sample causing rapid oxidation of the redox centers and uptake of protons. In this case the measured

amount of H^+ consumed was also lower than the sum of the redox centers in the oxidase undergoing oxidation. Re-reduction of the oxidase by ascorbate/Ru II was so slow compared to the rate of oxidation that the scalar release due to oxidation of ascorbate, which proceeded at a rate of $3 \cdot 10^{-3} \cdot \mathrm{s}^{-1} \cdot \mathrm{aa_3}^{-1}$ was negligible. The shortfall attributable to a redox Bohr effect amounted at pH 7.1 to a $\mathrm{H}^+/\mathrm{COX}$ ratio of 2.14 (Table 1, column B), which was practically equal to that obtained with succinate as reductant. Under these circumstances no significant 'P' and 'F' states of the oxidase [5] are expected to be formed upon oxygenation of the reduced oxidase both in the succinate and in the ascorbate plus RII experiments.

In order to determine the specific contributions of the four redox centers in the oxidase to the Bohr effect, experiments were carried out with cyanide-liganded oxidase, heme a_3 blocked in the oxidized
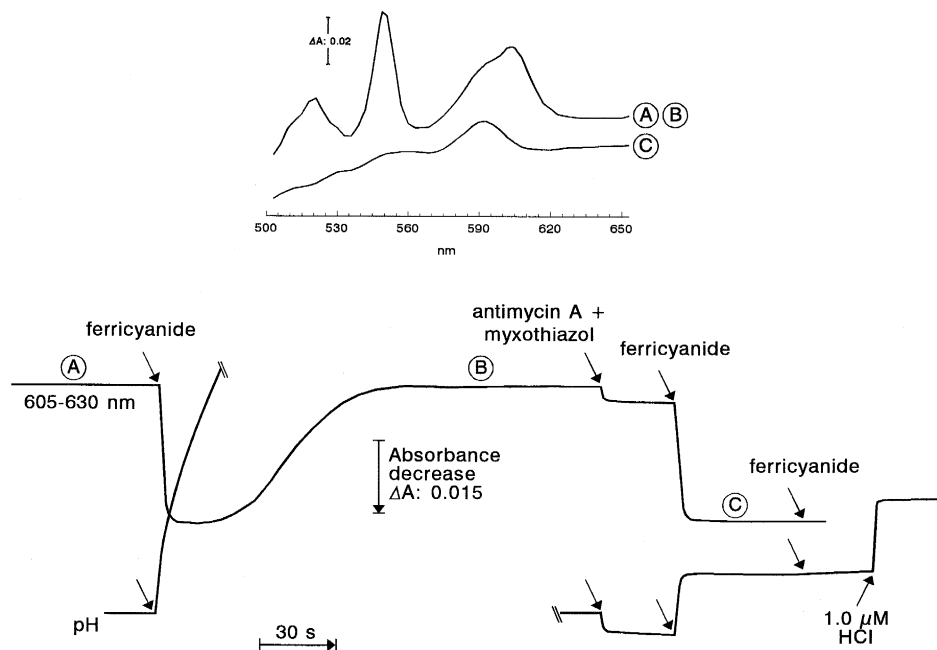

Fig. 3. Scalar H^+ transfer associated with oxidation of metal centers in CO-liganded cytochrome-c oxidase from the fully reduced to the mixed valence state. 1.2 μM cytochrome-c oxidase was suspended in 0.15 M KCl, 0.1 mM EDTA and supplemented with 2 μM cytochrome-c, 0.1 mg/ml broken rat liver mitochondria and 0.5 μg rotenone/ml (pH 7.3). The suspension was bubbled first for 2 min with nitrogen and then with CO. A combined pH electrode was inserted in the cuvette containing the CO-saturated suspension and this was then layered with mineral oil. Addition of 2 mM succinate caused formation of the fully reduced CO-liganded cytochrome-c oxidase in 10–15 min (spectrum A). Simultaneous recording of pH changes and heme a redox transitions, elicited upon successive additions of 25 μM ferricyanide, 0.1 μM antymicin A plus 0.3 μM myxothiazol and 12.5 μM ferricyanide, are presented. Spectrum C, recorded after the second addition of ferricyanide, shows the formation of the CO-liganded mixed valence oxidase.

form [38,40,41], or with CO-liganded oxidase, heme a_3 and Cu_B blocked in the reduced state [36]. With cyanide-liganded oxidase reduction by ferrocytochrome c of heme a, Cu_A (measured directly) and Cu_B, estimated from the difference between the equivalents of cyt c^{2+} oxidized and heme a and Cu_A reduced, (Table 1, column C) resulted in an uptake of protons. These were released in the same amounts upon oxidation of the metal centers by ferricyanide. The number of Bohr protons exchanged in this case per oxidase molecule undergoing oxido-reduction amounted to 0.66 at pH 7.2. Under these experimental conditions ferrocytochrome c did not fully reduce the metal centers in the oxidase (Table 1, column C). Moreover, due to the relatively high Em of the ferro-ferricytochrome c couple, increasing the concentration of ferrocytochrome c never gave complete reduction of the oxidase metal centers (data not shown). To overcome these difficulties reductants with more negative Em values were used. CN-liganded cytochrome-c oxidase in the presence of a stoicheiometric amount of cytochrome c was supplemented either with succinate in the presence of a catalytic amount of mitochondria (Fig. 2A), or with ascorbate plus TMPD (Fig. 2B). In both cases full

reduction of heme a, CuA (measured directly: see Table 1, columns D and E) and presumably Cu_B (cf. Ref. [36]) was achieved, resulting in a net release of protons. The amount of H^+ release was, however, less than that expected from the oxidation of the hydrogenated substrates by the oxidase and cytochrome c. The shortfall in H^+ release gives the number of Bohr protons taken up per oxidase molecule undergoing reduction, which amounted here to about 0.9 H^+/COX at pH 7.2.

In another set of experiments cytochrome-c oxidase was fully reduced by succinate plus a trace of broken mitochondria and cytochrome c in the presence of CO. Fig. 3 shows the characteristic spectrum generated by CO binding to the reduced enzyme (spectrum A). Addition of a small amount of ferricyanide, which oxidized heme a, Cu_A and cytochrome c (re-oxidation of Cu_B and heme a_3 was blocked by CO) generated the mixed valence oxidase (spectrum C). The oxidation of heme a and Cu_A was accompanied by a simultaneous, rapid H^+ release which preceded that arising from succinate oxidation by ferricyanide (Fig. 3). In about 2 min heme a and Cu_A were fully re-reduced by electrons from succinate. At this point antimycin plus myxothiazol were
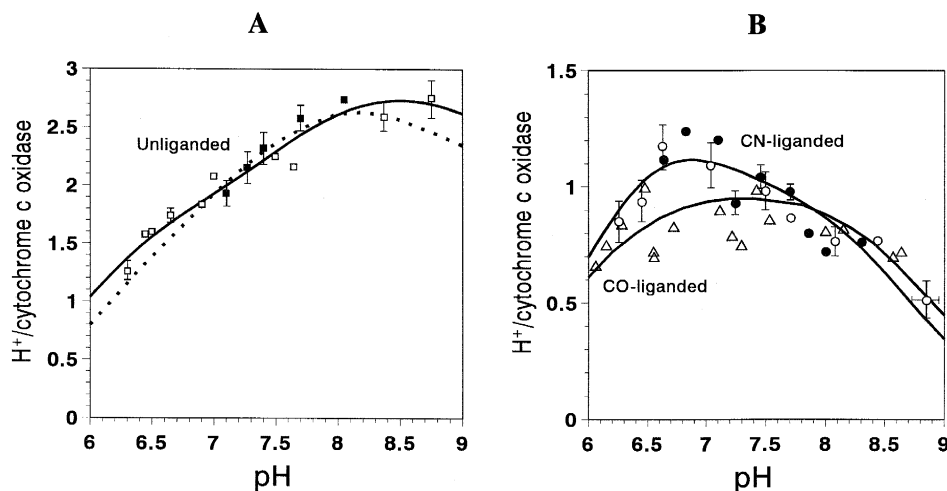

Fig. 4. Measured pH dependence of redox-Bohr effects (H^+/COX coupling number) in soluble cytochrome-c oxidase and the corresponding best-fit analyses. A: unliganded enzyme, open and closed squares refer to the experiments carried out using succinate or ascorbate as reductants respectively (the experimental details are presented in Fig. 1). Solid line: best-fit analysis of the experimentally determined H^+/COX ratios. Dotted line: curve constructed by using the means of the pK values obtained as described in Table 2C. B: CN-liganded enzyme; open and closed circles refer to experiments with succinate or ascorbate as reductants (see Fig. 2); triangles: CO-liganded enzyme (see Fig. 3). The curves represent the best fits obtained using the equation described in Section 2 and the pK values reported in Table 2A (see text for further details). Where indicated by vertical bars the points represent the mean ± S.E. of three or more H^+/COX measurements at the given pHs.

added to inhibit completely succinate oxidation. Addition of ferricyanide then gave full oxidation of heme a and Cu_A (not shown) which was accompanied by a H^+ release amounting to 0.76 H^+/COX, which presumably resulted from Bohr effect associated with the oxidation of heme a (as the Em of Cu_A is pH-independent [7,9,10]).

A systematic investigation of the H^+/COX ratios for redox-linked Bohr effects as a function of pH under the various experimental conditions illustrated above was then carried out. In the unliganded oxidase the experimental values obtained for the H^+/COX ratios increased from about 1 at pH 6 to a maximum of 2.7 at alkaline pH values (Fig. 4A). In the CN-liganded enzyme the H^+/COX ratios exhibited a bell-shaped pH profile with highest ratios of 1.1–1.2 at pH values close to 6.8 (Fig. 4B). This indicates

that Bohr effects in the CN-liganded enzyme involve 2 protolytic groups (see also Ref. [32]). In the CO-liganded enzyme the H^+/COX ratios also showed a bell-shaped pH profile but, in this case, the maximum H^+/COX ratios did not exceed 1.0 and were at pHs around neutrality lower than those measured in the CN-liganded enzyme (see also Ref. [32]).

The experimentally determined pH dependence of the H^+/COX ratios was then fitted with functions involving different numbers of protolytic groups (see Eq. (1) in Section 2). The experimental H^+/COX ratios with the CN-liganded oxidase (Cu_A, heme a and Cu_B redox active) could best be fitted by a function with 2 protolytic groups (Fig. 4B) whose respective pK_{ox} and pK_{red} values are given in Table 2A. The H^+/COX ratios measured in the CO-liganded enzyme (Cu_A and heme a redox active)

Table 2

pK values $\pm$ S.D. obtained from 'best-fit' analysis of the pH dependencies of the measured H^+/COX ratios for redox-linked scalar H^+ transfer in soluble cytochrome-c oxidase

(A)

Expt. conditions	Active redox centers	$pK_{ox} \pm$ S.D.	$pK_{red} \pm$ S.D.	Possible linkage
A Unliganded	a $Cu_A a_3 Cu_B$	6.0 ± 0.9	7.2 ± 0.6	Cu_B
		6.0 ± 0.9	9.4 ± 0.3	a
		7.2 ± 0.6	≥ 12.0	a_3
		7.7 ± 0.4	≥ 12.0	a_3
B CN-liganded	a $Cu_A Cu_B$	6.2 ± 0.2	6.8 ± 0.1	Cu_B
		6.1 ± 0.2	8.7 ± 0.1	a
C CO-liganded	a Cu_A	5.8 ± 0.1	8.9 ± 0.1	a

(B)

Expts.	Active redox centers	$pK_{ox} \pm$ S.D.	$pK_{red} \pm$ S.D.
A–B	a_3	6.8 ± 0.2	≥ 12.0
		7.4 ± 0.2	≥ 12.0
A–C	Cu_B	6.3 ± 0.2	7.3 ± 0.7
	a_3	7.4 ± 0.7	≥ 12.0
		7.4 ± 0.6	≥ 12.0
B–C	Cu_B	6.5 ± 0.2	7.0 ± 0.1

(C)

	Active redox centers	$pK_{ox} \pm$ S.D.	$pK_{red} \pm$ S.D.
	a	6.0 ± 0.2	8.8 ± 0.1
	a_3	7.1 ± 0.4	≥ 12.0
		7.4 ± 0.4	≥ 12.0
	Cu_B	6.4 ± 0.2	7.0 ± 0.3

The pK values are those obtained from the 'best-fit' analysis detailed under Section 2. Panel A refers to the pH dependence of the experimentally measured H^+/COX ratios presented in Fig. 4. Panel B shows the pKs used for the 'best-fit' analysis of the differences calculated as described in the legend to Fig. 5. Panel C gives the means of the pK values for the four groups calculated in B, C, A–B, A–C and B–C.
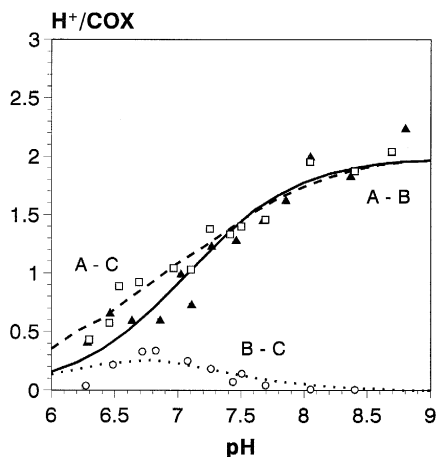
Fig. 5. pH dependence of the redox-Bohr effects in cytochrome-*c* oxidase. Identification of the redox centers responsible for redox-linked pK shifts was attempted by subtracting from the H^+/COX ratios that were experimentally measured in the unliganded oxidase (squares in Fig. 4A) those measured in the CN-liganded (circles in Fig. 4B; A–B) and in the CO-liganded oxidase (triangles in Fig. 4B; A–C). The subtraction of the H^+/COX ratios in the CO-liganded oxidase from those in the CN-liganded oxidase (B–C) is also presented. These calculation were made on the assumption that CN and CO binding do not affect in a significant way the pK shifts associated with oxido-reduction of the metal centers. The curves represent the 'best fit' analyses with the pKs reported in Table 2B.

could best be fitted by a function with 1 protolytic group (Fig. 4B, Table 2A). The pH dependence for the H^+/COX ratios in the unliganded oxidase could best be fitted by a function comprising contributions from 4 protolytic groups (Fig. 4A solid line, Table 2A).

The differences between the H^+/COX ratios measured in the unliganded enzyme and those measured in the CN-liganded enzyme (the putative contribution of heme a_3, Fig. 5A,B) could best be fitted by a function involving 2 redox-linked protolytic groups, whose pK values were essentially equal to those of 2 out of the 4 groups which gave the best fit for the unliganded enzyme (Table 2A,B). The differences between the H^+/e^- ratios in the unliganded and those in the CO-liganded enzyme (the putative contribution of heme a_3 and Cu_B, Fig. 5A–C) could best be fitted by a function with 3 protolytic groups whose pK values were almost equal to those of 3 out of the 4 groups giving the best fit curve for the unliganded enzyme (Table 2A,B). Two of the pKs giving the

curve A–C were also equal to those of the 2 groups of the curve A–B (Table 2A,B). At acidic pH the curve A–C has an upward deviation away from curve A–B (Fig. 5). Differences between the H^+/COX ratios in the CN-liganded enzyme (B) and the CO-liganded enzyme (C) (contribution of Cu_B, Fig. 5B,C) could best be fitted by a function with a single protolytic group whose pK_{ox} and pK_{red} were practically equal to those of 1 of the 4 groups postulated for the unliganded enzyme and 1 of the 3 groups required for the curve fitting the differences between the unliganded and the CO-liganded enzyme (curve A-C) (Table 2A,B).

The data thus allowed estimates of the number and the pK values of the protolytic groups specifically linked to redox transitions of the individual metal centers in the oxidase, namely heme a, heme a_3 and Cu_B. Two groups appear to be linked to redox transitions of heme a_3 and one each to redox transitions of heme a and Cu_B (see Table 2). A curve (dotted line in Fig. 4 A) for the pH dependence of the overall H^+/COX ratios constructed by using the means of the pK values obtained from the functions fitting the H^+/COX ratios in CN-liganded and CO-liganded oxidase and the differences between the unliganded and CN-liganded and CO-liganded oxidase (Table 2C), practically coincided with the curve obtained for the experimentally measured H^+/COX ratios in the unliganded enzyme (solid line, Fig. 4A). It should be noted that other 'best-fit' analyses, obtained using different numbers of protolytic groups and different pK values, failed to give equally satisfactory pattern for the number and pK values of protolytic groups linked to these redox-transitions.

4. Discussion

The redox Bohr effects [14–17] linked to hemes a and a_3 and Cu_B (the midpoint potential of Cu_A is pH-independent [7,9,10]) in cytochrome-*c* oxidase can be directly analyzed by following pH changes accompanying oxidation-reduction of the metal centers [16]. Previous measurements carried out in our [27–29] and in other laboratories [30–32] showed that 2–3 protons per oxidase molecule are released on oxidation of the fully reduced oxidase (and taken

up on its reduction). The present extension of these studies allowed us to: (i) identify 4 groups undergoing reversible pK shifts upon oxidation-reduction of the electron transferring metals in the enzyme; (ii) estimate the relative pK values and (iii) attribute the pK shifts of these groups to redox transitions of the individual metal centers. Two protolytic groups, with pK_{ox} and pK_{red} values around 7 and > 12 respectively result in being linked to redox transitions of heme a_3. One protolytic group with pK_{ox} and pK_{red} values around 6 and 7 respectively is apparently linked to redox transitions of Cu$_B$. Measurements of Mitchell and Rich [32] carried out over a limited pH range indicated the exchange of 2 protons per oxidase on oxido-reduction of the heme a_3-Cu$_B$ binuclear center, one of which was ascribed to Cu$_B$ [32] (see also Ref. [11]). Linkage of one protolytic group with pK shifts associated to oxido-reduction of heme a_3 was also suggested by Hallen et al. on the basis of kinetic data [42]. The fourth group identified here exhibits a pK shift from around 6.0 to 9.0 linked to oxido-reduction of heme a (cf. Ref. [32]). The assignment of the pK shifts of the four protolytic groups to individual metal centers in the oxidase does not exclude the possibility that the pK of a single group could also be influenced by cooperative interactions of different metals (cf. Ref. [11]). The correspondence between the pK values of the four groups as obtained in the free and the liganded oxidase (see Table 2) would, however, indicate that, if occurring, these interactions do not alter significantly the pK shifts arising from the individual linkages.

One of the two protolytic groups linked to heme a_3 could be a H_2O molecule ligated to the heme iron [44]. The other, or both, may be protolytic residues in subunit I of the oxidase or propionate substituents of the porphyrin ring of the heme. The pK values of the group linked to Cu$_B$ could identify it with one of three conserved histidines which serve as ligands for Cu$_B$ [24,25]. The group linked to heme a would be represented by a protolytic residue of subunit I in direct or indirect co-operative communication with the heme a group. Since the reduction potential of heme a is sensitive to the pH of the matrix space [8], the group linked to heme a is likely to exchange protons with the inner aqueous space (cf. Ref. [43]).

Heme a and the heme a_3-Cu$_B$ binuclear center are bound in subunit I to histidine residues of transmembrane helices 2, 10, 6 and 7, in regions extending toward the outer membrane surface. There, through a peripheral loop connecting helices 11 and 12, they come into contact with the C-terminal domain of subunit II holding the binuclear Cu$_A$ center [24,25].

It is generally thought that in order to be coupled to proton pumping electron flow in the oxidase must follow the sequence cyt. $c \rightarrow$ Cu$_A \rightarrow$ heme $a \rightarrow$ heme a_3-Cu$_B$ center [5,24,25,45]. Evidence has been obtained in one of our laboratories indicating that electron transfer from Cu$_A$ to the heme a_3-Cu$_B$ center can take place along two pathways [45] (see also Ref. [46]). The first electron travelling via heme a is proposed to be associated through short- and long-range co-operative linkages of redox transitions of this heme to an uptake of H^+ from the inner phase (cf. Ref. [8]) and its transfer to the heme a_3-Cu$_B$ binuclear center where subsequent proton pumping would be coupled to oxygen reduction to H_2O [5]. The heme a Bohr effect could thus allow coupling of proton translocation to oxygen reduction and permit proton pumping to occur at a maximal rate. The second redox pathway is proposed to involve direct electron transfer from Cu$_A$ to the binuclear center, bypassing heme a (cf. Ref. [25]), and thus a decoupling of the proton pump [45,46].

Genetic analysis of bacterial heme-cupper oxidases [47] and the recent X-ray crystallographic determination of the structure of cytochrome-c oxidase of Paracoccus denitrificans [24] and bovine heart [25], show that in subunit I there are various conserved protolytic residues in the proximity of heme a and the binuclear heme a_3-Cu$_B$ center, which could contribute to pK shifts linked to the redox transitions described here (cf. Ref. [48]). Amino acid residues distant from the redox centers, but still connected to these by polarizable hydrogen bonds [49], could also be involved in the redox Bohr effects [15,17,25]. In this respect the data obtained by the present approach could provide further clues when electrostatic calculations based on X-ray structures will become available.

Tsukihara et al. [25] have identified, by X-ray crystallography of the bovine heart oxidase, a hydrogen-bond interaction between heme a and subunit-I Ser382. This residue would be connected to a hydrogen-bond network, by which redox transitions of the heme could control proton pumping.

Work along the lines described in this paper, in combination with further mutational and X-ray crystallographic analyses of the three-dimensional structure of the oxidases in different redox states, should be able to identify the groups characterized here and allow us to elucidate the role that short and long range redox-linked pK shifts of protolytic residues, associated with co-operative mobility of protein domains (cf. Ref. [25]) may play in primary protonmotive redox catalysis and proton pumping by cytochrome-c oxidase (see Refs. [15,17]).

Acknowledgements

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