Coupling of Electron Transfer with Proton Transfer at Heme *a* and Cu_A (Redox Bohr Effects) in Cytochrome *c* Oxidase. Studies with the Carbon Monoxide Inhibited Enzyme

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ABSTRACT: A study is presented on the coupling of electron transfer with proton transfer at heme *a* and Cu_A (redox Bohr effects) in carbon monoxide inhibited cytochrome *c* oxidase isolated from bovine heart mitochondria. Detailed analysis of the coupling number for H^+ release per heme *a*, Cu_A oxidized (H^+/heme *a*, Cu_A ratio) was based on direct measurement of the balance between the oxidizing equivalents added as ferricyanide to the CO-inhibited fully reduced COX, the equivalents of heme *a*, Cu_A, and added cytochrome *c* oxidized and the H^+ released upon oxidation and all taken up back by the oxidase upon rereduction of the metal centers. One of two reductants was used, either succinate *plus* a trace of mitochondrial membranes (providing a source of succinate-c reductase) or hexaammineruthenium(II) as the chloride salt. The experimental H^+/heme *a*, Cu_A ratios varied between 0.65 and 0.90 in the pH range 6.0−8.5. The pH dependence of the H^+/heme *a*, Cu_A ratios could be best-fitted by a function involving two redox-linked acid−base groups with pK_o−pK_r of 5.4−6.9 and 7.3−9.0, respectively. Redox titrations in the same samples of the CO-inhibited oxidase showed that Cu_A and heme *a* exhibited superimposed E'_m values, which decreased, for both metals, by around 20 mV/pH unit increase in the range 6.0−8.5. A model in which oxido−reduction of heme *a* and Cu_A are both linked to the p*K* shifts of the two acid−base groups, characterized by the analysis of the pH dependence of the H^+/heme *a*, Cu_A ratios, provided a satisfactory fit for the pH dependence of the E'_m of heme *a* and Cu_A. The results presented are consistent with a primary involvement of the redox Bohr effects shared by heme *a* and Cu_A in the proton-pumping activity of cytochrome *c* oxidase.

Mitochondrial and prokaryotic cytochrome *c* oxidase have four redox centers (*1, 2*). A binuclear Cu_A center bound to subunit II, which titrates as one electron redox entity (*3*), is the entry port for the electrons delivered by cytochrome *c* (*1*). Cu_A transfer electrons, via a hydrogen bond/ion pair network of residues in subunits II and I, to heme a bound to subunit I (*4, 5*). Heme *a* transfers, in turn, electrons to the heme a_3−Cu_B binuclear center, also in subunit I, where dioxygen is reduced to H_2O with consumption of protons from the inner (N) aqueous phase (*1, 2*). Electron flow from cytochrome *c* to O_2 is, in addition, coupled to pumping of up to 1 H^+/e^- from the N to the outer (P) aqueous phase (*1, 6*−*8*).

Investigations on the mechanism of proton pumping have led, from time to time, to proposals that this process is directly linked to oxido−reduction of Cu_A (*9, 10*), heme *a* (*11*−*15*), and the binuclear center (*4, 16*). Search of the redox center(s) directly involved in proton pumping has, first of all, to take into account those which exhibit coupling of electron transfer with proton transfer (*17*). This thermodynamic linkage between oxido−reduction of metal centers and the protonation state of acid−base groups in cytochrome complexes, denominated redox Bohr effects (*18*) by analogy with the cooperative linkage phenomena in hemoglobin known as Bohr effects (*19*), results in pH dependence of the midpoint redox potential (decrease of E_m with increase of pH (*17, 20*)) and in net proton release/uptake by the enzyme associated with oxidation/reduction of the metals respectively (*21*).

In the unliganded mitochondrial cytochrome *c* oxidase the E_m's of both hemes *a* and a_3 show pH dependence (*11, 22*−*24*). The pattern of the pH dependence of the two hemes is, however, complicated by redox and protolytic interactions between them and with Cu_B (*24, 25*). Thus differences have been obtained in quantitative estimates of the pH dependence of heme *a* and a_3, as well as in the number of the putative acid−base groups involved (*23, 24*). In the presence of cyanide, which clamps heme a_3 in the oxidized state, the E_m of heme *a* and its pH dependence are still affected by interaction with Cu_B and possibly also Cu_A (*25, 26*). In the CO-liganded state both a_3 and Cu_B are clamped in the reduced state and heme *a* and Cu_A can be analyzed in the absence of interaction with Cu_B and heme a_3. Under these conditions the E_m of heme *a* is reported to exhibit a "small" pH dependence, with E'_m decrease of 10−20 mV per pH

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unit increase (*26, 27*). The E_m of Cu_A is generally considered to be pH independent (*28*), although a dependence of around −10 mV per pH unit has been reported (*23, 29*).

Direct analysis of the pH dependence of H^+ release/uptake associated with oxidation/reduction of the metal centers in cytochrome *c* oxidase isolated from bovine heart mitochondria (COX) allowed our group to characterize, in this way, the redox Bohr effects (*8, 30*). The H^+/COX linkage numbers measured in the pH range 6.0−8.5 could be best fitted with a function involving a minimum of four protolytic groups, each undergoing reversible p*K* increase in the oxidized/reduced/oxidized transitions of the redox centers in the unliganded oxidase (*30*). The four p*K* shifts were attributed to redox transitions of the individual centers from their correspondence with those obtained by best-fit analysis of the H^+/COX ratios measured in the CN-liganded and CO-liganded oxidase (*30*).

Here we present a detailed analysis of the H^+/e^- linkage in the purified CO-inhibited COX, measuring directly the balance between the oxidizing equivalents added (as ferricyanide) to the reduced oxidase, the equivalents of heme *a*, Cu_A, and added cytochrome *c* oxidized and the H^+ released upon oxidation, and all taken up back upon rereduction of COX. The experimental H^+/heme *a*, Cu_A ratio varied between 0.65 and 0.90 in the pH range 6.0−8.5 and could be best-fitted with a function involving two redox-linked acid−base groups. Redox titrations carried out on the same samples of CO-inhibited COX showed that heme *a* and Cu_A exhibited superimposed E_m values with the same pH dependence and a slope of around −20 mV/pH unit increase in the 6.0−8.5 range. A model is proposed in which oxido−reductions of heme *a* and Cu_A are both linked to the protonation state of two common acid−base groups, resulting in the observed "small" pH dependence of their redox potentials. These observations together with the measured H^+/heme *a*, Cu_A ratios, of about 0.8 at pH's near neutrality, are consistent with a primary involvement of the redox Bohr effect shared by heme *a* and Cu_A in the proton-pumping activity of cytochrome *c* oxidase.

MATERIALS AND METHODS

Enzyme Preparation. Cytochrome *c* oxidase was purified from beef heart mitochondria as described in ref *31* or in ref *32*. In both preparations the nanomoles of heme $a + a_3$/mg of protein were about 10 and SDS−PAGE analysis revealed the complete set of 13 subunits (*33*). The activity of the enzyme preparations (measured polarographically in 40 mM KCl, 10 mM Hepes (pH 7.4), 0.1 mM EDTA, 0.1% dodecyl maltoside, 50 μM cytochrome *c*, and 40 nM aa_3, supplemented with 25 mM ascorbate plus 200 μM TMPD) was around 80 O_2 molecules$\cdot s^{-1}\cdot aa_3^{-1}$ (i.e., 320 TN/s).

Measurements of pH and Redox Changes. Simultaneous recordings of absorbance and pH changes were carried out with a diode-array spectrophotometer and a combined electrode respectively with accuracy of 5×10^{-4} absorbance and 10^{-3} pH unit (overall response time < 1 s). The diode-array spectrophotometer was used in the multiwavelength mode so that simultaneous recordings of pH and difference absorbance changes were carried out. The wavelengths selected, 550−630, 590−630, 605−630, and 800−710 nm, were used to determine cytochrome *c*, heme a_3−CO, heme *a*, and Cu_A, respectively. The mutual optical overlapping among cytochrome *c*, heme a_3−CO, and heme *a* was removed by solving the following matrix:

$$A_{550-630} = [\text{cyt } c](19.1) + [\text{heme } a_3\text{−CO}](3.81) + [\text{heme } a](-0{,}25)$$

$$A_{590-630} = [\text{cyt } c](-1.11) + [\text{heme } a_3\text{−CO}](9.75) + [\text{heme } a](6.03)$$

$$A_{605-630} = [\text{cyt } c](-0.36) + [\text{heme } a_3\text{−CO}](2.85) + [\text{heme } a](21.73)$$

The differential extinction coefficients are from ref *34*; the absorbance change at 800−710 nm was corrected for the contribution of cytochrome *c* ($\Delta\epsilon = 0.36$ mM^{-1}), and a $\Delta\epsilon = -1.0$ mM^{-1} was used for Cu_A (*35*). Ferricyanide was assayed at 420−500 using a $\Delta\epsilon$ of 1.0 mM^{-1}.

Data Analysis. The pH dependence of the observed redox-linked H^+-transfer reactions, expressed as H^+/heme *a*, Cu_A ratios, was best fitted with curves obtained using eq 1, which

$$H^+/\text{heme } a, Cu_A = \sum i|(1/(1 + 10^{pH-pK_{ox}})) - (1/(1+10^{pH-pK_{red}}))| \quad (1)$$

gives the theoretical pH dependence of the H^+/COX ratio for redox-Bohr effects attributable to protolytic group(s) with different p*K*'s (pK_o and pK_r) in the oxidized and reduced state.

Redox Titration of CO-Liganded Cytochrome c Oxidase. Oxidative titration of fully reduced CO-liganded cytochrome *c* oxidase *plus* cytochrome *c* was performed by addition of small amounts of freshly prepared and anaerobic solutions of ferricyanide; reductive back-titration was performed with Na dithionite solutions. After each addition, once equilibration was achieved, the spectrum from 500 to 800 nm was recorded. The spectrum in the presence of an excess of oxidant (pure mixed valence state) was taken as reference and subtracted from the spectra recorded during the titration. The absorbances at 605−630 nm and 550−540 nm were corrected for the mutual optical overlapping between cytochrome *c* and heme *a* (*26*) and that at 800−710 nm (Cu_A) for the contribution of cytochrome *c*. The E_h was estimated from the redox levels of cytochrome *c*, whose E_m is pH-independent in the range explored (*26*). Simulations of the pH dependence of E'_m was carried out using eqs 4−6 in the text, which describe the pH dependence of one or two redox centers with the assumption that their oxidoreductions are linked to the (de)protonation of separate and/or common protolytic group(s) (see text and ref *17*).

Materials. Horse heart cytochrome *c* (type VI), antimycin A, and myxothiazol were from Sigma Chemical Co., hexaammineruthenium(II) chloride was from Aldrich, and potassium ferricyanide was from BDH Chemicals Ltd. All other reagents were of the highest purity grade commercially available.

RESULTS

Measurements of H^+ Transfer Associated with Oxido−Reduction of CO-Inhibited COX. COX, saturated with CO, was reduced by succinate by means of a trace of broken mitochondria and cytochrome *c*. Succinate oxidation con-

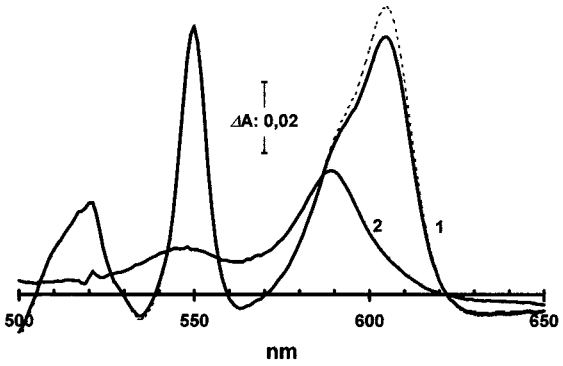

FIGURE 1: Analysis of spectral changes associated with oxido−reduction of metal centers in CO-liganded cytochrome *c* oxidase. 3.5 μM purified bovine cytochrome *c* oxidase was suspended in 0.15 M KCl and 0.1 mM EDTA and supplemented with 3.5 μM cytochrome *c*, 0.2 mg/mL broken beef heart mitochondria, and 0.5 μg of rotenone/mL (pH 7.2). The suspension was bubbled first with nitrogen and then with pure CO. Addition of 2 mM succinate to the CO-saturated COX solution covered by a layer of deareated mineral oil caused formation of the fully reduced CO-liganded cytochrome *c* oxidase in 10−15 min (spectrum 1). Spectrum 2 shows formation of the pure mixed-valence state elicited upon addition of ferricyanide (within 2−3 s). Rereduction of heme *a* (and Cu_A) by the succinate−mitochondria system resulted in about 1 min in restoration of spectrum 1. When rereduction by succinate was completely inhibited by the presence of antimycin A *plus* myxothiazol, the addition of ferricyanide resulted in the formation of a stable (for minutes) mixed-valence compound (spectrum 2). The dotted line represents the spectrum obtained upon addition of Na dithionite.

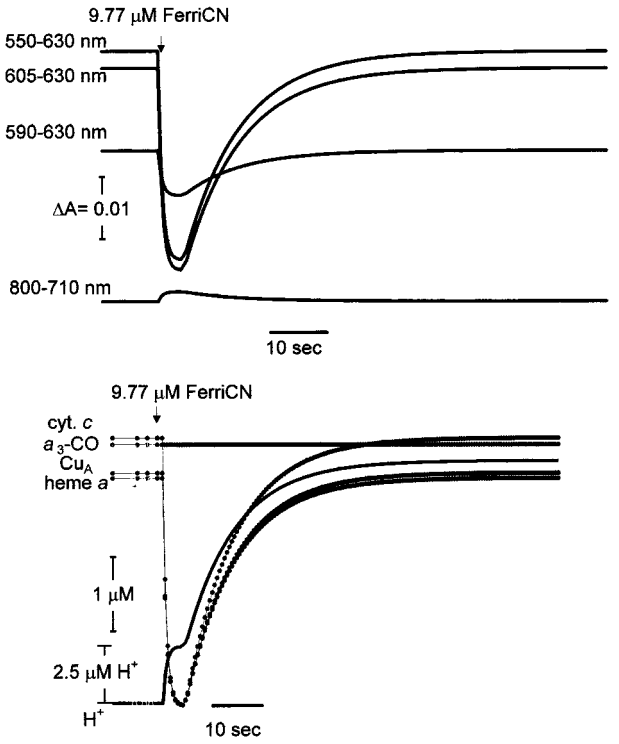

FIGURE 2: Measurements of scalar H^+ transfer associated with oxidation−reduction of Cu_A and heme *a* in CO-liganded cytochrome *c* oxidase. The experimental conditions were those described in the legend to Figure 1. A combined pH electrode was inserted in the cuvette containing the CO-saturated suspension, and this was then layered with deaerated mineral oil. The upper traces show the absorbance changes associated with redox transitions of the metal centers elicited upon addition of ferricyanide, starting from the fully reduced CO-liganded cytochrome *c* oxidase obtained by the addition of succinate (see spectrum 1 in Figure 1). The lower traces show pH changes and the results of spectral deconvolution made to remove the mutual optical overlapping of cytochrome *c*, heme a_3−CO, and heme *a* (see under Materials and Methods). The rereduction of the metal centers by the succinate−mitochondria system was slowed by adding enough malonate in order to separate kinetically it from the rapid oxidation phase (see spectrum 2 in Figure 1). The rapid pH change associated with the oxidation phase gave an H^+/heme *a* ratio of 0.75; the slow acidification, synchronous with the rereduction of the centers by succinate, added to the initial H^+ release resulted in an H^+/ferricyanide ratio of 1.00.

sumed all the oxygen in the COX solution, which was isolated from the air by a layer of mineral oil. Figure 1 shows the characteristic spectrum of the reduced CO-inhibited COX (spectrum 1). Addition of an amount of ferricyanide equivalent to 90% of the sum of reduced heme *a*, Cu_A, and cytochrome *c* rapidly oxidized these centers (oxidation of heme a_3 and Cu_B was blocked by CO) generating the mixed-valence oxidase (spectrum 2). Figure 2 shows that the oxidation of heme *a* and Cu_A was accompanied by synchronous release of 0.75 H^+/heme *a*, Cu_A (at pH 7.2). This was followed after a short lag, by further H^+ release during rereduction of heme *a*, Cu_A, and cytochrome *c* by succinate. The overall reduction of ferricyanide by succinate resulted, as expected, in a 1 to 1 stoichiometric net H^+ release (see Tables 1 and 2). Thus any proton release associated with oxidation of the redox centers in the oxidase (cytochrome *c* is irrelevant in this respect since its E_m is pH independent in the range explored here (*26*)) is fully reversible.

Figure 3 shows the second step of the experiment presented in Figure 2; 3−5 min after full rereduction of heme *a*, Cu_A, and cyt *c* by succinate, antimycin plus myxothiazol was added to inhibit completely succinate oxidation. Addition of the same amount of ferricyanide used in the absence of these inhibitors gave permanent oxidation of cyt *c*, Cu_A, and heme *a*. This was accompanied again by rapid release of 0.75 H^+/heme *a*, Cu_A.

To verify whether some of the rapid H^+ release observed upon oxidation of CO-inhibited COX by ferricyanide could be contributed by oxidation of ubiquinol or redox Bohr effects (*21*) in the trace of broken mitochondria used to produce anaerobic reduction of CO-inhibited COX, the experiments illustrated in Figures 1−3 were repeated varying the amounts of broken mitochondria or cytochrome *c* used as reducing system. The results presented in Table 1 show that the extent of H^+ release/heme *a*, Cu_A, measured upon oxidation of the CO-inhibited oxidase by ferricyanide, did not vary, either in the absence or in the presence of antimycin plus myxothiazol, when the amounts of mitochondria or cytochrome *c* added were changed by 2−4-fold.

The possibility of contributions to the H^+ release, associated with the oxidation of CO-inhibited reduced COX by ferricyanide, from oxidation of components of broken mitochondria, decomposition and oxidation of the (a_3^{2+}•Cu_B^+)−CO complex by ferricyanide, or slow reduction of ferricyanide by reactions 2 and 3 were eliminated by the

$$CO + H_2O + 2Fe^{3+}(CN)^-_6 \rightarrow CO_2 + 2Fe^{2+}(CN)^-_6 + 2H^+ \quad (2)$$

$$CO_2 + H_2O \rightarrow HCO_3^- + H^+ \quad (3)$$

result of a set of experiments, as those illustrated in Figures 1−3, in which the equivalents of cyt *c*, Cu_A, and heme *a*

Table 1: Effect of the Concentration of Cytochrome *c* and Mitochondria on the H^+-Transfer Associated with Oxidation−Reduction of Cu_A and Heme *a* in CO-Liganded Cytochrome *c* Oxidase[a]

CO−aa_3 (μM)	BHM (mg/mL)	cyt *c* (μM)	ant. A myx	H^+_R/ heme *a*, Cu_A	$H^+_{R(F)}$/ ferricyanide
3.5	0.1	3.5	−	0.93	0.98
			+	0.61	
3.5	0.2	3.5	−	0.83	1.10
			+	0.81	
3.5	0.4	3.5	−	0.79	0.98
			+	0.58	
3.5	0.2	7.5	−	0.80	1.00
			+	0.80	
0	0.2	3.5	−	0	1.00
			+	0	

[a] The experimental conditions are those described under Materials and Methods and in the legend to Figures 2 and 3, with pH 7.2. The last line shows the results of an experiment where cytochrome *c* oxidase was absent. BHM: beef heart mitochondria. H^+_R and $H^+_{R(F)}$ are proton release associated with oxidation of the redox centers in the oxidase and final proton release upon re-reduction of the oxidized centers by succinate (see Figures 2 and 3).

oxidized by a known amount of ferricyanide and the H^+ released were accurately measured at various pH's. The results of these experiments, summarized in Table 2, show that, upon addition to CO-inhibited reduced COX of an amount of ferricyanide corresponding to 80−85% of reduced cyt *c*, Cu_A, and heme *a*, the sum of the oxidation extents of these three centers corresponded precisely to the oxidizing equivalents added, either when calculated from the transient oxidation in the absence of antimycin and myxothiazol or from the permanent oxidation obtained in the presence of these inhibitors. It can be noted that reduction by succinate of the CO-saturated COX converted all heme a_3 to the a_3^{2+}−CO compound and that the addition of ferricyanide did not cause any change of this adduct, thus converting COX to its CO-liganded mixed-valence state. These results show, unequivocally, that the only components oxidized by ferricyanide were, under the experimental conditions used, added cyt *c*, Cu_A, and heme *a* of the CO-inhibited COX, thus excluding the possibility of any significant oxidation of the trace of ubiquinol in the small amount of broken mitochondria used for the reducing process, as suggested by Verkhovsky et al. (*36*), CO, and the (a_3^{2+}·Cu_B^+)−CO adduct. pH measurements showed, under these conditions, the release, associated to oxidation of Cu_A and heme *a*, of 0.7 H^+/heme *a*, Cu_A both in the absence and in the presence of antymicin plus myxothiazol. The accuracy of the measurement of the H^+/heme *a*, Cu_A ratio was internally verified by the ratio of the overall H^+ release to the amount of ferricyanide finally reduced by succinate in the absence of antimycin plus myxothiazol, which amounted, as expected, to one. The H^+/heme *a*, Cu_A ratio was also measured in experiments in which these redox centers were reduced by hexaammineruthenium(II) in CO-inhibited COX under the conditions used by Verkhovsky et al. (*36*). The H^+/heme *a*, Cu_A ratios so obtained coincided with those measured under the conditions illustrated in Figure 3 and Table 2 (see Figure 4).

A set of experiments was then carried out to examine the influence of pH on the H^+/heme *a*, Cu_A ratio for the H^+ release associated to oxidation by ferricyanide of Cu_A^+ and heme a^{2+} in the CO-inhibited COX reduced by succinate or by hexaammineruthenium(II). Figure 4 shows that the H^+/heme *a*, Cu_A ratios, measured in the pH range 6.0−8.5, varied between 0.65 and 0.90. Analysis of the means of the H^+/heme *a*, Cu_A for the various pH's, measured in the succinate- or hexaammineruthenium(II)-reduced CO−COX, showed that these could be best-fitted ($\chi^2 = 1.8 \times 10^{-3}$) with a function (eq 1) involving two redox-linked acid−base groups with pK_o−pK_r of 5.4−6.9 and 7.3−9.0, respectively (Figure 4, solid line). A function with one redox-linked acid−base group, which we previously favored on the basis of a more limited number of measurements (*30*), gave comparatively a less satisfactory fit ($\chi^2 = 1.8 \times 10^{-2}$) (Figure 4, dashed line).

It should, however, be noted that the redox-linked p*K* shifts attributed here to two protonable groups could also reflect the involvement of a core of more than two electrostatically interacting acid−base groups (*38*).

Redox Titrations of Cu_A and Heme a in the CO-Inhibited Cytochrome c Oxidase. CO-saturated COX was brought to full anaerobic reduction by succinate with cytochrome *c* and a trace of broken mitochondria under the same conditions used to measure H^+ transfer. After addition of antimycin plus myxothiaziol, to block further electron transfer from the succinate−mitochondria system, oxidative titration was performed by ferricyanide additions and reductive back-titration by Na dithionite additions using cytochrome *c* as redox marker. The experimental E′m of Cu_A and heme a, measured at various pH's in the range 6.0−8.5, exhibited practically the same values (Figure 5). Linear regression analysis of the experimentally determined E'_m values (dotted line) gave for both heme *a* and Cu_A a decrease of 16 mV/pH unit increase in the range 6.0−8.5. At pH 7.5 both Cu_A and heme *a* exhibited an E'_m of around 250 mV (Figure 5, dotted line).

DISCUSSION

The present study defines the quantitative parameters of the coupling of electron transfer with proton transfer (redox Bohr effects) at heme *a* and Cu_A, analyzed in the CO-inhibited, cytochrome *c* oxidase isolated from bovine heart mitochondria. Direct determinations were made of the stoichiometric ratios of oxidizing equivalents (added as ferricyanide to CO-inhibited reduced COX) to the equivalents of heme *a*, Cu_A, and added cytochrome *c* oxidized and to the equivalents of protons released upon oxidation, the latter all being taken back up by COX upon rereduction. These experiments showed unequivocally that oxido−reduction of heme *a* and Cu_A is thermodynamically coupled to a net H^+ transfer, with an H^+/heme *a*, Cu_A ratio which varies between 0.65 and 0.90 in the pH range 6.0−8.5.

The present extensive determination of the H^+/heme *a*, Cu_A ratios, carried out on CO−COX reduced by two different systems, i.e., either by succinate plus a trace of mitochondrial membranes (providing a source of succinate−cytochrome *c* reductase) (*30*) or directly by hexaammineruthenium(II), which is a pure electron donor (*36*), gave experimental ratios, clearly resulting from at least two acid−base groups linked to oxido−reduction of the metal centers. A function with these two linked acid−base groups with pK_o and pK_r of 5.4−6.9 and 7.3−9.0, respectively, gave a satisfactory best fit ($\chi^2 = 1.8 \times 10^{-3}$) of the experimental means of the H^+/heme *a*, Cu_A ratio measured in the pH range 6.0−8.5.

Table 2: Statistical Analysis of the Ferricyanide-Induced Redox Transitions of the Metal Centers in the CO-Liganded Reduced Cytochrome *c* Oxidase and Associated H^+ Transfer[a]

ant. A plus myx	1: ferriCN added	2: cyt *c* oxidized	3: heme *a* oxidized	4: Cu_A oxidized	2 + 3 + 4	1/(2 + 3 + 4)	a_3−CO	a_3−CO + ferriCN	H^+_R/ heme *a*, Cu_A	$H^+_{R(F)}$/ ferriCN
−	9.71	3.68	3.08	3.22	9.98	0.97	3.93	3.87	0.73	1.01
	±0.27	±0.12	±0.08	±0.14	±0.27	±0.02	±0.26	±0.25	±0.07	±0.02
+	9.22	3.52	2.94	2.89	9.35	0.99	3.90	3.85	0.71	
	±0.23	±0.05	±0.11	±0.01	±0.14	±0.02	±0.12	±0.12	±0.05	

[a] The experimental conditions are those described in the legends of Figures 2 and 3 (pH 7.2). The values (±SEM) indicate μM concentrations of cytochrome *c*, heme *a*, and Cu_A that are oxidized upon addition of ferricyanide; a_3−CO and a_3−CO + ferriCN indicate the concentrations of the a_3^{2+}−CO compound, before and after the addition of ferricyanide, respectively, when the stoichiometry measurements were made. H^+_R and $H^+_{R(F)}$ are proton release associated with oxidation of the heme *a* and Cu_A and final proton release upon re-reduction of the oxidized centers by succinate. The concentration of both COX and cytochrome *c* was 3.8−4.0 μM.

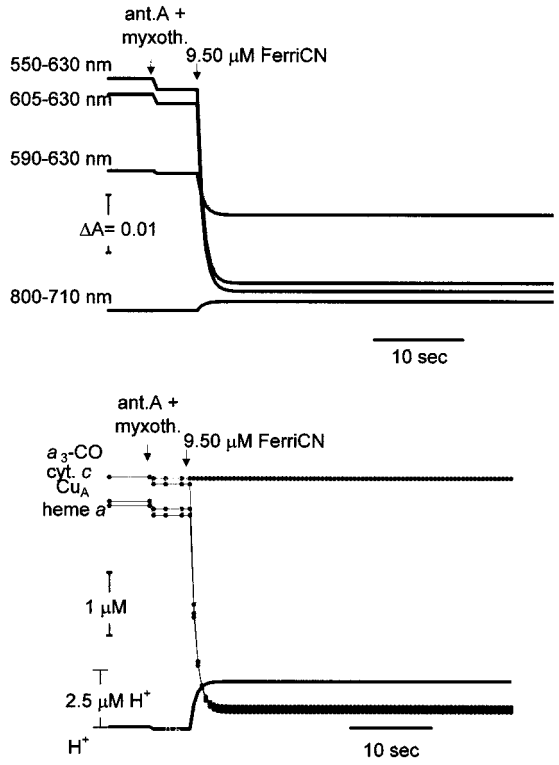

FIGURE 3: Measurement of scalar H^+ transfer associated with oxidation of Cu_A and heme *a* in CO-liganded cytochrome *c* oxidase from the reduced to the mixed-valence state. The experimental conditions were those described in the legend to Figure 1. The CO-liganded enzyme was fully reduced by succinate, supplemented with 0.1 μM antymicin A plus 0.3 μM myxothiazol then and pulsed with ferricyanide. The upper traces show the absorbance changes associated with oxidation of the metal centers elicited upon addition of ferricyanide; the lower traces show pH changes and the results of spectral deconvolution carried out as described under Materials and Methods. The rapid pH change associated with the oxidation of Cu_A and heme *a* gave an H^+/heme *a* ratio of 0.75.

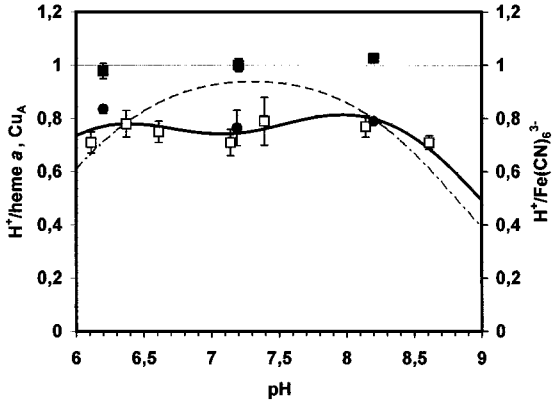

FIGURE 4: Influence of pH on H^+ release associated with oxidation by ferricyanide of heme a^{2+} and Cu_A^+ in soluble CO-liganded cytochrome *c* oxidase. The experimental conditions were those described in the legend to Figures 1−3. Black squares: ratios of the total amount of protons released, at the end of the rereduction by succinate of metal centers, with respect to the ferricyanide added (see Figure 2). White squares: H^+/heme *a*, Cu_A ratios measured from the acidification accompanying oxidation of heme *a* and Cu_A in the succinate−mitochondria reduced CO−COX (antymicin A plus myxothiazol present). Black circles: H^+/heme *a*, Cu_A ratios measured from the acidification accompanying oxidation of heme *a* and Cu_A in the hexaammineruthenium(II)-reduced CO−COX. The experimental conditions for the measurements using hexaammineruthenium(II) as reductant were the following: 2.5 μM aa_3 plus 0.25 μM cytochrome *c* was saturated with CO and supplemented with successive additions of hexaammineruthenium(II) (around 0.2 mM final concentration), until the reduced aa_3 CO-liganded species was obtained. Oxidation (around 90%) of cytochrome *c*, heme *a*, and Cu_A was achieved by adding a slight excess of ferricyanide (1.5-fold with respect to the sum of the equivalents of reduced cytochrome *c*, heme *a*, and Cu_A). The vertical bars represent the mean ± SEM of four or more H^+/heme *a*, Cu_A measurements at the given pH's. Extension of the measurements at pH values lower than 6.0 or higher than 9.0 has been avoided because of inactivation of the enzyme at these extreme pH's (see also ref *23*). The curves represent the best fit obtained using the equation described under Materials and Methods with one (dashed line), $pK_o = 5.8$ and $pK_r = 8.8$, or two (solid line) acid−base groups, pK_o−pK_r of 5.4−6.9 and 7.3−9.0, respectively (see text).

Verkhovsky et al. (*36*) recently reported an H^+/aa_3 ratio at pH 7.2 of 0.3 (changing to 0.43 at pH 6.6 and to 0.20 at pH values above 7.5) for H^+ release upon stepwise oxidation by ferricyanide of CO-inhibited hexaammineruthenium(II)-reduced COX. However this paper did not contain a complete parallel time course of the pH changes observed and the oxidation of heme *a*, nor data on the oxidation of Cu_A. This makes evaluation of the results (Verkhovsky et al. (*36*)) difficult. But it can be noted that these authors measured the H^+/aa_3 ratios using consecutive additions of substoichiometric amounts of ferricyanide to the same sample of CO-inhibited COX. We have now found that, upon addition of substoichiometric amounts of ferricyanide to CO-inhibited COX, H^+/aa_3 ratios similar to those of Verkhovsky et al. (*36*) are obtained. However simulation of such H^+/aa_3 ratios based upon a calculation of the statistical distribution of COX molecules in various states of heme *a* and Cu_A oxidation (N.C., G.C., and S. P., results to be presented elsewhere) indicate that such lower ratios can be a consequence of the coupling of two groups undergoing p*K* shifts to both heme *a* and Cu_A, as in the model which is discussed below. Our approach to measure the H^+/heme *a*, Cu_A ratios, going from fully reduced redox centers to their almost complete oxida-

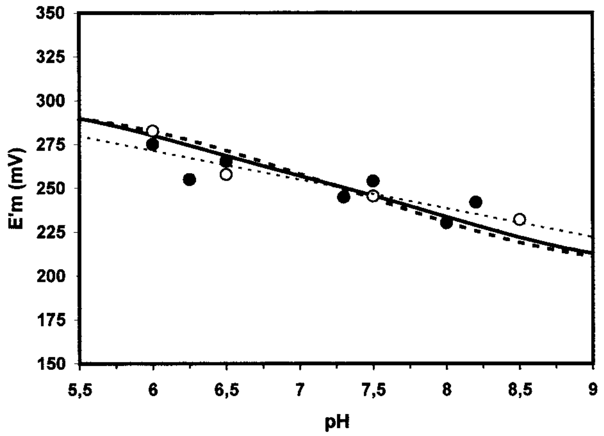

FIGURE 5: Redox titration of heme *a* and Cu_A in the CO-liganded cytochrome *c* oxidase. 3−4 μM cytochrome *c* oxidase saturated with CO was suspended in 0.1 M potassium phosphate, at the pH's shown, supplemented with 3−4 μM cytochrome *c* and 0.2 mg/mL of mitochondria. After addition of 2 mM succinate, which caused almost full reduction of both heme *a* and Cu_A, antimycin A plus myxothiazol was added to block further electron transfer from the succinate−mitochondria system, and oxidative titration was performed by addition of small amounts of freshly prepared and anaerobic solutions of ferricyanide; reductive back-titration was performed with Na dithionite solutions. After each addition, once equilibration was achieved, the spectrum from 500 to 800 nm was recorded and analyzed as described under Materials and Methods. Black circles: E'_m of heme *a*. White circles: E'_m of Cu_A. Dotted line: linear regression analysis of the E'_m of heme *a* and Cu_A. The two curves represent the mathematical simulation (eq 4) assuming one (dashed line) or two (solid line) different acid−base group(s) linked to both heme *a* and Cu_A. The pK_o-pK_r values, used in the simulations, were those obtained from the analysis of the pH dependence of the H^+/heme *a*, Cu_A ratio, i.e., 5.8−8.8 for a single acid−base group or 5.4−6.9 and 7.3−9.0 for two different acid−base groups (see Figure 4); E_m was 297 mV. See text for further explanations.

tion, with the addition of an amount of ferricyanide equivalent to 90% of the sum of the reduced centers, avoids, on the other hand, this complication and gives the maximal H^+/heme *a*, Cu_A coupling ratios attainable.

In previous papers (*15, 30, 37*) we too had attributed the redox-coupled H^+ transfer to heme *a* alone, as the available data indicated the E_m of Cu_A to be pH independent (*28*; but see refs *23* and *29*). Electron/proton coupling at heme *a* only would, we agree, conflict with previous measurements of its E_m between pH 6 and 8. These have shown a pH dependence in the CO-inhibited COX much smaller than the −60 mV/pH unit increase expected for a protonation-linked oxido−reduction (*26, 27*). We have now directly analyzed the influence of pH on the E_m of heme *a* and Cu_A, by means of titrations using the redox state of cytochrome *c* as a marker, in the same CO-inhibited COX samples as were used to measure H^+ transfer. The results of these titrations revealed two important features: (i) the E_m values of heme *a* and Cu_A are essentially the same at all the pH examined in the range 6.0−8.5; (ii) the E_m of heme *a*, and hence also that of Cu_A, decreased as the pH was increased from 6.0 to 8.5 with a slope close to −16 mV/pH.

The observation of the parallel redox patterns of heme *a* and Cu_A provides a clue to resolve the apparent conflict between the measured H^+/heme *a*, Cu_A ratios, for redox-coupled H^+ transfer and the small pH dependencies of the two redox centers. The experimental values for the E'_m of heme *a* and Cu_A measured in the pH range 6.0−8.5, giving by linear regression (Figure 5, dotted line) a decrease of 16 mV/pH unit increase for both heme *a* and CuA, could be best fitted (Figure 5, solid line) by eq 4, which applies for

$$E'_m = E_m + 30(\log(10^{-2\mathrm{pH}} + 10^{-(\mathrm{p}K_{r1}+\mathrm{pH})} + 10^{-(\mathrm{p}K_{r1}+\mathrm{p}K_{r2})}) - \log(10^{-2\mathrm{pH}} + 10^{-(\mathrm{p}K_{o1}+\mathrm{pH})} + 10^{-(\mathrm{p}K_{o1}+\mathrm{p}K_{o2})})) \quad (4)$$

linkage of each of the two protolytic groups identified from the analysis presented in Figure 4 to oxido−reduction of both heme *a* and Cu_A, as shown in eq 5. It can however be

$$2\ \text{cyt.}\ c^{2+} + \underset{[\mathrm{X\ Y}]}{Cu_A^{2+}\text{---}a^{3+}} + (m+n)H^+ \leftrightarrow 2\ \text{cyt.}\ c^{3+} + \underset{[\mathrm{XH^+_m\ YH^+_n}]}{Cu_A^{+}\text{---}a^{2+}} \quad (5)$$

deduced from the general formulation of eq 4, as given in Clark (*17*), that functions with one (Figure 5, dashed line) or more than two such acid−base groups, each associated to oxido−reduction of both heme *a* and Cu_A, generate best-fit curves for the E'_m of the two redox components (heme *a* and Cu_A) (not shown), practically superimposable with the curve obtained with only two postulated protonable groups, all the theoretical curves having slopes of −20 mV or less *per* unit pH increase. Linkage of protolytic group(s) separately to heme *a* or Cu_A did not give, on the contrary, satisfactory fits with the experimental E'_m values exhibited by heme *a* and Cu_A in the pH range explored (not shown).

The linkage of both Cu_A and heme *a* with common acid/base group(s) in the oxidase results, under equilibrium redox titration conditions, in a pH dependence of the E_m values markedly smaller than −30 mV/pH unit. But such acid/base group(s) can nevertheless display a full coupling activity in the unliganded membrane-bound oxidase in the respiring steady state. Under these conditions electrons pass one way in sequence through Cu_A and then heme *a*. Evidence has been obtained indicating that both CO-inhibited and unliganded COX, when reconstituted in liposomes (*37*), show vectorial proton/electron coupling (redox Bohr effects) at heme *a*. The present work extends this idea to involvement of Cu_A too in this coupling. Protons are taken up from the inner (N) aqueous space upon reduction and released in the outer (P) phase upon oxidation (*8, 37*).

From studies of pH effects on redox behavior of heme *a* in cyanide-liganded COX Moody and Rich (*25*) as well as Mitchell (*26*) have concluded that heme *a* and Cu_B share a redox link with the protonation state of an acid/base group. It is therefore possible that oxido−reduction of heme *a* is linked through protolytic interactions both to the oxido−reduction of Cu_A, on the cytochrome *c* side, and to the oxidoreduction of Cu_B, on the oxygen side, in the proton-motive redox cycle. It may thus play a central role in the proton-pumping activity of cytochrome *c* oxidase. Such a role of Cu_A and heme *a* in proton pumping is not necessarily alternative to a role of the heme a_3-Cu_B center (*4, 16, 39, 40*). The low- and high-potential redox centers may, in fact, cooperate in the proton pumping activity of the oxidase (*15*). The recent observation that part of the proton pumping may take place in the reductive phase of the oxidase catalytic cycle (*41*; see also ref *42*) appears to be consistent with the

possibility that electron flow through Cu_A−heme *a* represents, at least, one of the coupling steps in the proton pump of cytochrome *c* oxidase (*15*; see also refs *9*−*14*).

A structural basis for the protolytic interaction of heme *a* and Cu_B is suggested by X-ray analysis (*42*). Hydrogen bond/ion pair network of conserved residues of subunits II and I were identified in the crystal structure of both bovine (*5*) and *Paracoccus denitrificans* (*4*) cytochrome *c* oxidase providing a pathway for electron transfer between Cu_A and heme *a*. This underlines the close structural and functional interaction of the Cu_A and heme *a* domains. In their X-ray analysis of oxidized and reduced crystals of the bovine oxidase, Yoshikawa et al. (*43*, *44*) have described a redox-linked conformational change of a segment from Gly49 to Asn55 of subunit I located at the P surface close to subunit II. On the basis of this structural change, these authors have proposed a role in proton pumping of Asp51 of subunit I and Ser205 of subunit II.

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