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Proton Transfer Reactions Associated with the Reaction of the Fully Reduced, Purified Cytochrome *c* Oxidase with Molecular Oxygen and Ferricyanide†

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ABSTRACT: A study is presented on proton transfer associated with the reaction of the fully reduced, purified bovine heart cytochrome *c* oxidase with molecular oxygen or ferricyanide. The proton consumption associated with aerobic oxidation of the four metal centers changed significantly with pH going from ≈3.0 H^+/COX at pH 6.2−6.3 to ≈1.2 H^+/COX at pH 8.0−8.5. Rereduction of the metal centers was associated with further proton uptake which increased with pH from ≈1.0 H^+/COX at pH 6.2−6.3 to ≈2.8 H^+/COX at pH 8.0−8.5. Anaerobic oxidation of the four metal centers by ferricyanide resulted in the net release of 1.3−1.6 H^+/COX in the pH range 6.2−8.2, which were taken up by the enzyme on rereduction of the metal centers. The proton transfer elicited by ferricyanide represents the net result of deprotonation/protonation reactions linked to anaerobic oxidoreduction of the metal centers. Correction for the ferricyanide-induced pH changes of the proton uptake observed in the oxidation and rereduction phase of the reaction of the reduced oxidase with oxygen gave a measure of the proton consumption in the reduction of O_2 to $2H_2O$. The results show that the expected stoichiometric proton consumption of $4H^+$ in the reduction of O_2 to $2H_2O$ is differently associated, depending on the actual pH, with the oxidation and reduction phase of COX. Two H^+/COX are initially taken up in the reduction of O_2 to two OH^- groups bound to the binuclear Fe a_3−Cu_B center. At acidic pHs the third and fourth protons are also taken up in the oxidative phase with formation of $2H_2O$. At alkaline pHs the third and fourth protons are taken up with formation of $2H_2O$ only upon rereduction of COX.

Cytochrome *c* oxidase (COX),[1] the heme copper terminal oxidase in the respiratory chain of mitochondria and plasma membrane of various prokaryotes, catalyzes the reduction of dioxygen to water by ferrocytochrome *c* (reaction 1).

$$4(\text{cytochrome } c^{2+}) + 4H^+ + O_2 + nH^+_{in} \rightarrow 4(\text{cytochrome } c^{3+}) + 2H_2O + nH^+_{out} \quad (1)$$

Reaction 1 is coupled to the translocation of up to four protons across the coupling membrane from the inner to the outer (cytochrome *c* side) aqueous phase (*1*−*5*). In the past few years definite progress has been made in the elucidation of the mechanism by which cytochrome *c* oxidase catalyzes the reduction of O_2 to H_2O (*3*, *6*−*9*). This has been

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[1] Abbreviations: COX, cytochrome *c* oxidase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine.

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contributed by results of advanced time-resolved spectroscopic analysis (*3*), mutational analysis of the prokaryotic oxidases (*7*), and X-ray crystallographic resolution of the atomic structure of bovine (*10*, *11*) and prokaryotic (*12*−*14*) cytochrome *c* oxidase. A catalytic cycle for cytochrome *c* oxidase was proposed by Wikstrom in the 1980s (*15*; see also ref *3*), which incorporated two intermediates, P and F, he had discovered in the reverse electron flow from H_2O to COX (*16*) and the fully reduced enzyme. The cycle consists of a reductive phase in which the four redox centers of the oxidized enzyme (Cu_A, heme *a*, heme a_3, and Cu_B) are reduced by cytochrome *c*, followed by an oxidative phase, in which dioxygen is reduced to two water molecules by the fully reduced enzyme. According to eq 1 four protons per COX should be consumed upon aerobic oxidation of the fully reduced enzyme. It was, however, found in our (*17*−*19*) and other laboratories (*20*−*22*) that at pHs of 7.4−8.0 no more than two protons per COX, both in mitochondria and in the isolated state, were taken up when the fully reduced enzyme was oxidized by O_2. Shortfall in the measured versus the expected proton uptake was explained as a consequence of concomitant proton release from protolytic groups in the enzyme due to decrease of their p*K*'s associated with oxidation of the metal centers (*17*−*19*) (redox Bohr effect) (*23*) or of the production, in the oxidative phase of the catalytic cycle, of incompletely protonated water molecules: one hydroxyl group and one water molecule (8) or two hydroxyl groups (*22*, *24*) bound to the heme a_3−Cu_B binuclear center, in part counteracted by release of Bohr protons.

In this paper the proton transfer associated, over a wide pH range, with the oxidation of the fully reduced purified COX, in the soluble state, by dioxygen and its subsequent rereduction were measured and compared with the proton transfer observed on anaerobic oxidation of reduced COX by ferricyanide. The latter gave a measure of the net result of deprotonation/protonation reactions linked to anaerobic oxidoreduction of the metal centers in COX. The stoichiometric consumption of $4H^+$ in the reduction of O_2 to $2H_2O$ was found to be differently associated, depending on the actual pH, with the oxidation and reduction phase of COX. The results are discussed in terms of proton transfer associated with the catalytic cycle of O_2 reduction in COX.

MATERIALS AND METHODS

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

Enzyme Preparation. Cytochrome *c* oxidase was purified from beef heart mitochondria as described in ref *25*. The nmol of heme $a + a_3$/mg of protein was about 10, and SDS−PAGE analysis revealed the complete set of 13 subunits (*26*). The activity of the enzyme preparation [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, at room temperature, around 80 O_2 molecules$\cdot s^{-1}\cdot aa_3^{-1}$.

Measurements of pH and Redox Changes. Simultaneous recording of absorbance and pH changes was carried out with a diode array spectrophotometer (settled in the multiwavelength mode) and a combined electrode, respectively, with accuracy of 5×10^{-4} absorbance and 10^{-3} pH unit (overall response time <1 s) (*18*). The wavelengths selected were 550−540 nm to determine cytochrome *c* and 445−470 and 605−630 nm to determine heme a_3 and heme *a*. In the latter the mutual optical overlapping between hemes *a* and a_3 in the α and γ region of the spectra was removed by solving the equations [contribution of cytochrome *c* was negligible, given the low concentration used (see legends to the Figures)]:

$$A_{445-470} = 70.21[\text{heme } a] + 82.37[\text{heme } a_3]$$

$$A_{605-630} = 20.31[\text{heme } a] + 5.59[\text{heme } a_3]$$

The differential extinction coefficients are from ref *27*. Cu_A redox changes were monitored at 800−710 nm using $\Delta\epsilon = -1.0\ mM^{-1}$ (*28*). The redox changes of Cu_B were assumed to be equivalent to those of heme a_3. Ferricyanide was assayed at 420−500 nm using a $\Delta\epsilon$ of 1.0 mM^{-1}.

RESULTS

The four metal centers of a 1.0 μM sample of soluble bovine heart cytochrome *c* oxidase (COX) at pH 8.15 were fully reduced in anaerobiosis by succinate using a trace of broken mitochondria and cytochrome *c* (Figure 1A). Reoxidation of the reduced COX was then effected by the addition of O_2. Redox transitions of cytochrome *c*, hemes *a* and a_3, and associated proton transfer reactions were measured spectrophotometrically and potentiometrically, respectively, on the same sample.

Reduction by succinate of the metal centers of oxidized COX resulted in the release in the medium of 1.8 μM H^+ instead of 4 μM H^+ as expected from reaction 2 (plus an additional 0.5 μM H^+ due to reduction of added ferricytochrome *c*).

$$2(\text{succinate}) + n(\text{cyt } c^{3+}) + Cu_A^{2+}Fe_a^{3+}Fe_{a3}^{3+}Cu_B^{2+} \rightarrow 2(\text{fumarate}) + n(\text{cyt } c^{2+}) + Cu_A^{1+}Fe_a^{2+}Fe_{a3}^{2+}Cu_B^{1+} + (4+n)H^+ \quad (2)$$

$$n(\text{cyt } c^{2+}) + Cu_A^{1+}Fe_a^{2+}Fe_{a3}^{2+}Cu_B^{1+} + O_2 + (4+n)H^+ \rightarrow n(\text{cyt } c^{3+}) + Cu_A^{2+}Fe_a^{3+}Fe_{a3}^{3+}Cu_B^{2+} + (2+n/2)H_2O \quad (3)$$

Thus the reduction of the four metal centers in the oxidized COX was associated, at pH 8.15, with the uptake of 2.7 H^+/COX. Aerobic oxidation of the fully reduced COX resulted in the uptake of 1.7 μM H^+ instead of the 4 μM H^+ expected from reaction 3 (plus an additional 0.5 μM H^+ due to oxidation of ferrocytochrome *c*). Thus the oxidation of reduced COX resulted in the consumption of only 1.2 H^+/COX. This summed up with the 2.7 H^+/COX taken up upon reduction resulted in the consumption of 4 H^+/COX as expected from the overall reaction of reduction of O_2 to $2H_2O$. Upon O_2 exhaustion the excess of succinate began to rereduce the oxidized metal centers in the oxidase, and as

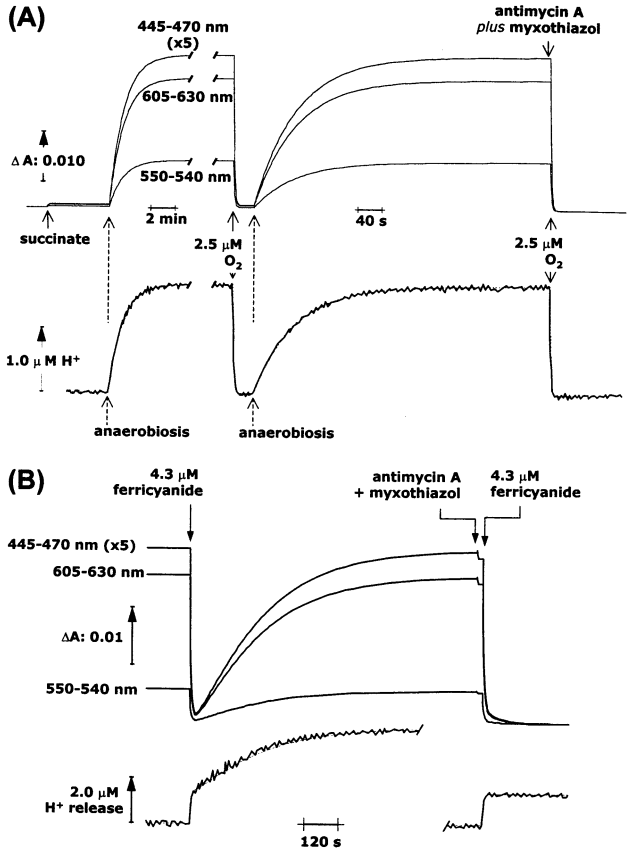

FIGURE 1: Measurement of scalar proton transfer associated with the reduction−oxidation phases of the metal centers in soluble cytochrome *c* oxidase. (A) 1.0 μM bovine purified cytochrome *c* oxidase was suspended in 0.15 M KCl and 0.1 mM EDTA (pH 8.15) and supplemented with 0.5 μM cytochrome *c*, 0.1 mg/mL frozen−thawed, broken beef heart mitochondria, and 0.5 μM rotenone. Simultaneous recordings of absorbance and pH changes were carried out in a plugged gastight cuvette as described under Materials and Methods. Where indicated, 5 mM succinate was added and the oxidase suspension left to reach anaerobiosis. After addition of 2 mM anaerobic malonate, to slow electron transfer from succinate, an amount of air-equilibrated water, containing a slight excess of equivalents of O_2 with respect to the reduced metal centers, was added. Following the oxidation rereduction cycle, 0.1 μM antymicin A plus 0.3 μM myxothiazol were added, and the oxygen pulse was repeated. The pH changes accompanying redox transitions of the metal centers in COX were calibrated with anaerobic titrated HCl solution. (B) The experimental conditions are as in (A) but with 0.3 μM cytochrome *c* present and pH 7.2. Upon achievement of anaerobiosis by succinate respiration (not shown), oxidation of the fully reduced metal centers of COX was elicited by addition of concentrated anaerobic ferricyanide solution, stoichiometric with the reduced metal centers. Following the oxidation rereduction cycle, 0.1 μM antymicin A plus 0.3 μM myxothiazol were added, and the ferricyanide pulse was repeated. The H^+/COX ratios reported in the text and Figure 2 were obtained by dividing the nequiv/mL of H^+ transfer, corrected for that associated with oxidoreduction of cytochrome *c* by the amount of COX undergoing oxidoreduction. The latter was estimated from the measurements of the specific redox changes of hemes *a* and a_3. For other details see Materials and Methods.

expected, from the sum of reactions 2 and 3, at the end of the anaerobic rereduction the pH returned to the value recorded before addition of O_2.

Once the redox centers in COX were fully rereduced by succinate, antimycin A plus myxothiazol was added to block cytochrome *c* reductase, and permanent complete oxidation of COX was effected by a further oxygen pulse (Figure 1A). This confirmed that complete oxidation of the metal centers was already achieved upon the oxygen pulse of reduced COX in the absence of the two inhibitors. The permanent oxidation of the reduced metal centers of COX (plus added cytochrome *c*) resulted in a permanent H^+ uptake of 1.7 μM H^+, which corresponded to the consumption of only 1.2 H^+/COX as observed upon oxidation of the metal centers of COX in the absence of the two inhibitors.

It has been previously observed in our laboratory that the number of H^+ consumed per COX in the aerobic oxidation of the fully reduced enzyme increased as the pH of the medium was decreased at values around and slightly below neutrality (*19*; see, however, refs *20*−*22*). An extensive series of experiments such as those illustrated in Figure 1A were thus carried out using succinate or ascorbate plus hexaammineruthenium as reducing substrates at various pHs in the range from 6.2 to 8.5. The results summarized in Figure 2 (panel A) show that the H^+ uptake/COX ratio for proton consumption in the aerobic oxidation of the fully reduced COX decreased significantly with pH going from 2.65 at pH 6.25 to 1.20 at pH 8.0−8.25 (open symbols). The H^+ uptake per COX measured in the reduction of the oxidized enzyme increased with pH going from 1.35 at pH 6.25 to 2.80 at pH 8.0−8.25 (panel A, closed symbols). The sum of the H^+ uptake measured in the oxidation and reduction phase, respectively, corresponded to the expected overall consumption of $4H^+$ in the reduction of O_2 to $2H_2O$.

In another set of experiments pH changes associated with anaerobic oxidation of the fully reduced COX by a stoichiometric amount of ferricyanide, i.e., in the absence of oxygen reduction chemistry, were measured. The results presented in Figure 1B show that the oxidation of the metal centers of COX by ferricyanide was accompanied by synchronous H^+ release from the enzyme. This was followed by slower H^+ release associated with rereduction of the metal centers by succinate (catalyzed by the trace of mitochondria and cytochrome *c*), which was completely suppressed when succinate cytochrome *c* reductase was inhibited by myxothiazol plus antimycin A. Upon COX rereduction the final H^+ release was equivalent to that expected from succinate oxidation by the ferricyanide added; i.e., the protons released from COX were taken up by the enzyme. The H^+/COX ratio for the H^+ release upon oxidation by ferricyanide of the metal centers of cytochrome *c* oxidase amounted to 1.3−1.6 in the pH range 6.2−8.2 (Figure 2, panel B).

Addition to the proton uptake, observed in the aerobic oxidative phase of reduced COX, of the proton release elicited by anaerobic ferricyanide oxidation of COX gave H^+/COX ratios for proton consumption in the reduction of O_2 to $2H_2O$ which varied from ≈4 at pH 6.3 to ≈2.5 at pH 8.2 (Figure 2, panel C, open circles). Subtraction from the proton consumption, observed in the reductive phase of the aerobic experiments, of the H^+ transfer promoted by ferricyanide gave H^+/COX ratios which increased with pH from ≈0 at pH 6.3 to ≈1.5 at pH 8.2 (closed circles), thus complementing to 4 the proton consumption measured in the oxidative phase.

DISCUSSION

The X-ray crystallographic structures of bovine (*11*) and bacterial (*13*) COX reveal in the oxidized state in the enzyme

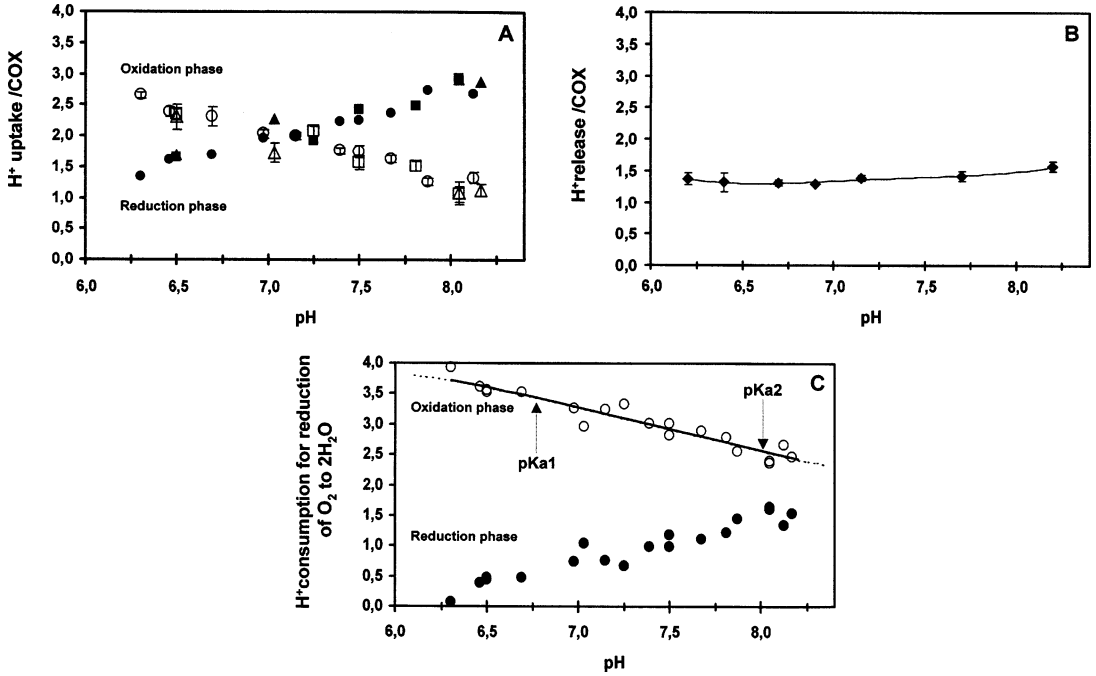

FIGURE 2: pH dependence of the proton transfer associated with oxidoreduction of metal centers in cytochrome *c* oxidase. (A) pH dependence of the H^+/COX ratios for proton uptake associated with aerobic oxidation and rereduction of metal centers in COX. Open circles and squares refer to the H^+ uptake/COX ratios associated with oxidation of COX by slightly over-stoichiometric or substoichiometric additions of O_2, respectively (in both cases succinate was the reducing substrate); the H^+/COX ratios measured in the absence or in the presence of 0.1 μM antimycin A plus 0.3 μM myxothiazol (see Figure 1A) were averaged for each measurement. Experimental conditions are those of Figure 1A. Open triangles: H^+ uptake/COX ratios associated with oxidation of COX by slightly substoichiometric additions of O_2 with ascorbate as reducing substrate. 1.0 μM cytochrome *c* oxidase was suspended in the same medium of the experiment in Figure 1A, supplemented with 0.1 μM cytochrome *c* and 5 mM ascorbate plus 100 μM hexaammineruthenium. Anaerobiosis was attained in 5−10 min, and the fully reduced cytochrome *c* oxidase plus cytochrome *c* was oxidized by the addition of a slightly substoichiometric amount of O_2 equivalents with respect to reduced metal centers. Closed symbols: H^+/COX ratios for proton uptake in the reduction by succinate of the oxidized enzyme. These ratios represent the difference between the expected and the observed H^+ release/COX in the reduction of the metal centers by succinate (see also Figure 1 and text). Each value is the mean of at least three different measurements ± SEM. (B) pH dependence of the H^+/COX ratio for proton release associated with ferricyanide oxidation of the metal centers in cytochrome *c* oxidase in the presence of antymicin A plus myxothiazol. The values reported are the means at each pH of at least four different determinations ± SEM, and the fitting line is the result of the polynomial equation $y = 0.156x^4 - 4.532x^3 + 49.342x^2 - 238.37x + 432.29$. (C) pH dependence of proton consumption in the reduction of O_2 to $2H_2O$ by COX. Open circles are obtained by adding to the H^+uptake/COX ratios measured in the conversion of COX from the fully reduced to fully oxidized state (panel A) the H^+ release/COX ratios measured in the anaerobic oxidation by ferricyanide at the corresponding pHs as obtained from the polynomial curve equation presented in panel B (see Table 1). The thick line represents the best fit of the corrected resulting values for the proton uptake associated with oxidation of fully reduced cytochrome *c* oxidase by O_2 obtained by the equation $H^+/COX = -4 + [10^{pH-pK_1}/(10^{pH-pK_1} + 1) + 10^{pH-pK_2}/(10^{pH-pK_2} + 1)]$ with $pK_1 = 6.75$ and $pK_2 = 8.02$. Closed circles are obtained by subtracting from the H^+ uptake/COX ratios measured in the conversion of the COX from the fully oxidized to fully reduced state (panel A) the H^+/COX ratios measured for the reversible proton release/uptake in the anaerobic oxidoreduction promoted by ferricyanide at the corresponding pHs as obtained from the polynomial curve equation presented in panel B. For other details see Materials and Methods and Figure 1.

Table 1: H^+ Consumed in the Reduction of O_2 to $2H_2O$ in the Transition from the Fully Reduced to the Fully Oxidized Cytochrome *c* Oxidase[a]

pH	H^+/COX	SEM	pH	H^+/COX	SEM
6.30	3.92	0.05	7.50	2.83	0.12
6.46	3.62	0.08	7.50	3.01	0.09
6.50	3.52	0.20	7.68	2.89	0.05
6.50	3.57	0.09	7.81	2.79	0.08
6.69	3.52	0.15	7.88	2.55	0.06
6.98	3.26	0.06	8.05	2.39	0.18
7.03	2.96	0.15	8.05	2.37	0.13
7.15	3.24	0.05	8.13	2.66	0.08
7.25	3.33	0.08	8.17	2.47	0.09
7.39	3.01	0.05			

[a] The values reported are those reported as empty circles in Figure 2C.

a continuous electron density between heme a_3 iron and Cu_B, which indicates the presence between these metal centers of two oxygen atoms. It has, in fact, been inferred from crystallographic data that in the oxidized state the space between the two metals is occupied by a peroxide ligand (*11*) or, alternatively, by a Cu_B-bound OH^- (or H_2O) and a water as the sixth heme a_3 iron ligand (*13*). This latter possibility appears to be supported by EXAFS and ENDOR spectroscopy (*29*) as well as by electrostatic simulations (*8*, *30*). No ligand has, on the other hand, been detected between Fe a_3 and Cu_B in the crystal structure of the reduced COX (*11*).

Time-resolved spectroscopic analysis of the reaction of O_2 with the fully or partially reduced COX, following flash photolysis of its complex with CO, has revealed the rapid formation of P and F intermediates, preceding formation of the oxidized enzyme (*8*, *31*). The same type of analysis showed that the overall conversion of COX from the fully reduced to the oxidized state, taking place in less than 1 ms after flash photolysis of the CO−COX complex, was associated in this time interval with a proton uptake of 1.3−

1.6 H^+/COX in the pH range 6.5−8.5 (*22*). These measurements were obtained from complex spectral changes of COX suspensions supplemented with different pH indicators. The shortfall in the expected consumption of 4 H^+/COX in the aerobic oxidation of the fully reduced COX (see also refs *17*, *19*, and *20*) has been taken as reflecting the consumption of two (*22*) or three (*8*) H^+/COX in the oxidation phase of the fully reduced enzyme, the remaining protons being taken up in the rereduction phase. Contribution to the shortfall of concomitant proton release by Bohr effects linked to the metal centers was also taken into account (*22*; see also refs 17−19).

The present results, which are based on direct measurements with a glass electrode, of the final balance of pH changes in the water bulk phase show that the expected stoichiometric consumption of four protons in the reduction of O_2 to $2H_2O$ is differently associated, depending on the actual pH, with the oxidation and the reduction phase of COX. In the oxidation phase the proton consumption decreases from ≈4 at pH 6.3 to ≈2.5 at pH 8.0−8.2. The proton consumption in the rereduction phase, practically zero at pH 6.3, increased to ≈1.5 at pH 8.0−8.2. The sum of the two phases of proton uptake resulted, at all the pH examined, in the expected consumption of four protons in the reduction of O_2 to $2H_2O$ (see Figure 2, panel C). The experimental points obtained for the proton consumption in the oxidation phase could be best fitted by an equation based on two p*K*'s of 6.7 and 8.0, respectively. It can be noted that p*K*'s in this range have been estimated for water associated with the binuclear center (cf. refs *30* and *31*).

Our results indicate that the H^+ consumption in the reduction of O_2 to $2H_2O$ by reduced COX proceeds in two steps (Figure 3). In the first the oxidation of the fully reduced metal centers results, via the formation of the P_R (or P_M) and F intermediates, in an oxidation state (O_I) in which two OH^- groups are bound to the oxidized metals of the binuclear center. The uptake of the first two protons utilized up to this stage is a fast process which is completed in the <1 ms interval monitored in the flow-flash analysis (*20*, *22*). This rapid uptake of the first two protons could be pH independent (*22*). At alkaline pHs neither of the two OH^- bound to the binuclear center, with respective p*K*'s of 6.7 and 8.0, can be further protonated to H_2O, unless they are released in the bulk phase upon rereduction of COX (cf. ref *22*), as also indicated by the absence of the density of oxygen in the space between Fe a_3 and Cu_B in the crystal of the reduced form of bovine COX (*11*). At acidic pHs the two OH^- bound to the binuclear center can, instead, be protonated to two H_2O molecules already in the oxidative phase. This uptake of the third and fourth proton could be a rather slow process. It might have been missed in the rapid flow-flash analysis (*22*) but not when enough time is allowed for full proton equilibration between the environment of the binuclear center and the bulk phase, as is the case of the measurements presented in this paper. The translocation to the binuclear site of the third and fourth proton could represent the rate-limiting step for the overall activity of the oxidase and might explain the marked pH dependence of the turnover, which exhibits maximum values at acidic pH (*35*).

The proton release associated to anaerobic oxidation by ferricyanide of the fully reduced unliganded COX, completely reversible on rereduction of the enzyme, represents

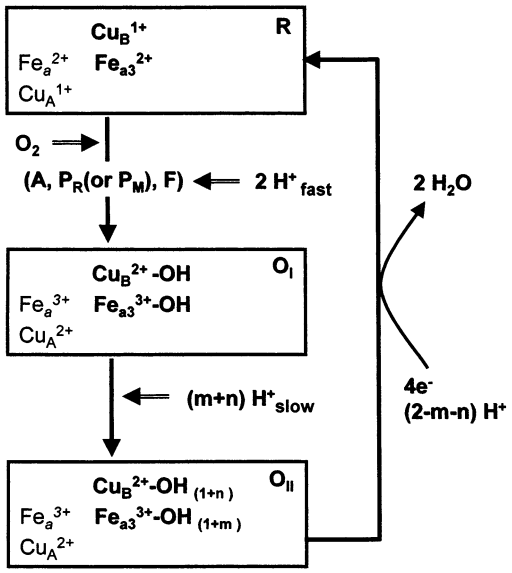

FIGURE 3: Proton consumption steps in the O_2 reduction by cytochrome *c* oxidase. The four metal centers in cytochrome *c* oxidase are shown in the boxes. R state refers to the fully reduced oxidase, and O_I and O_{II} refer to two different protonation states of the fully oxidized oxidase. It should be noted that the conversion of the oxidase from the R to the O_I state goes through intermediates A, P_R (or P_M), and F (see refs *39* and *40*). *n* and *m* refer to the fractional protonation state of the two OH^- bound to the binuclear center. Their value depends on the actual pH, being $(n + m) = 2$ at relatively acidic pH (i.e., $2H_2O$ molecule) or $(n + m) = 0$ at relatively alkaline pH (i.e., $2OH^-$ bound) (see Figure 2, panel C, and text).

the net result of deprotonation/protonation reactions linked to oxidoreduction of the metal centers, in the absence of binding and reduction chemistry of O_2. This proton transfer varied between 1.3 and 1.6 in the pH range 6.2−8.2. It is significantly higher and exhibits a different pH dependence profile than that previously found, in the same pH range, to be associated to oxidoreduction of heme *a* and Cu_A in the CO-inhibited COX (heme a_3 and Cu_B clamped in the reduced state), which amounted to 0.6−0.8 H^+/COX (28). This indicates that oxidoreduction of the four redox centers in the unliganded enzyme, in addition to the Bohr effects coupled to the oxidoreduction of heme *a* and Cu_A (*28*), is associated with additional protonation/deprotonation reactions linked to oxidoreduction of the binuclear center.

Evidence has been presented elsewhere for the involvement of the redox Bohr effects coupled to heme *a* and Cu_A in the proton pump of aa_3 cytochrome *c* oxidase (*36*−*38*). The protonation/deprotonation reactions linked to the anaerobic oxidoreduction of the binuclear center, evidence of which is already provided by the complex pH dependence of the midpoint redox potential of the metal centers of COX (*41*, *42*), can involve positive (H^+ binding upon reduction) and negative (H^+ release upon reduction) Bohr effects (*11*, *43*) as well as protonation/deprotonation of OH^-/H_2O ligands at the binuclear center (*44*).

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