pH Dependence of Proton Translocation in the Oxidative and Reductive Phases of the Catalytic Cycle of Cytochrome *c* Oxidase. The Role of H_2O Produced at the Oxygen-Reduction Site†

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ABSTRACT: A study is presented on the pH dependence of proton translocation in the oxidative and reductive phases of the catalytic cycle of purified cytochrome *c* oxidase (COX) from beef heart reconstituted in phospholipid vesicles (COV). Protons were shown to be released from COV both in the oxidative and reductive phases. In the oxidation by O_2 of the fully reduced oxidase, the H^+/COX ratio for proton release from COV (R → O transition) decreased from ≈2.4 at pH 6.5 to ≈1.8 at pH 8.5. In the direct reduction of the fully oxidized enzyme (O → R transition), the H^+/COX ratio for proton release from COV increased from ≈0.3 at pH 6.5 to ≈1.6 at pH 8.5. Anaerobic oxidation by ferricyanide of the fully reduced oxidase, reconstituted in COV or in the soluble case, resulted in H^+ release which exhibited, in both cases, an H^+/COX ratio of 1.7−1.9 in the pH range 6.5−8.5. This H^+ release associated with ferricyanide oxidation of the oxidase, in the absence of oxygen, originates evidently from deprotonation of acidic groups in the enzyme cooperatively linked to the redox state of the metal centers (redox Bohr protons). The additional H^+ release (O_2 versus ferricyanide oxidation) approaching 1 H^+/COX at pH ≤ 6.5 is associated with the reduction of O_2 by the reduced metal centers. At pH ≥ 8.5, this additional proton release takes place in the reductive phase of the catalytic cycle of the oxidase. The H^+/COX ratio for proton release from COV in the overall catalytic cycle, oxidation by O_2 of the fully reduced oxidase directly followed by re-reduction (R → O → R transition), exhibited a bell-shaped pH dependence approaching 4 at pH 7.2. A mechanism for the involvement in the proton pump of the oxidase of H^+/e^- cooperative coupling at the metal centers (redox Bohr effects) and protonmotive steps of reduction of O_2 to H_2O is presented.

Cytochrome *c* oxidase, the terminal heme-copper oxidase of the respiratory chain of mitochondria and various prokaryotes, catalyzes the reduction of O_2 to H_2O by ferrocytochrome *c* (reaction 1).

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

The redox reaction generates a transmembrane electrochemical proton gradient (Δp) (*1*). This first results from the consumption of "chemical" protons from the inner (N) aqueous space in the reduction of O_2 to H_2O by ferrocytochrome *c* located at the outer (P) side of the membrane (*2*, *3*). In addition, reaction 1 is coupled to the pumping of up to four protons (1 H^+ per e^- flowing from ferrocytochrome *c* to oxygen) from the N to the P aqueous phase (*1*, *4*). The oxidase has four redox centers: a binuclear Cu_A center, titrating as one electron redox entity, which is bound to subunit II, heme *a*, heme a_3, and Cu_B, all these bound to subunit I (*5*). Cytochrome *c* delivers electrons to Cu_A; heme *a* transfers electrons from Cu_A to the heme a_3−Cu_B binuclear center, where O_2 is reduced to H_2O (*1*, *5*, *6*).

Although the proton pump of the oxidase is a matter of intensive investigation, its molecular mechanism is still elusive (*6*−*9*). Different groups have developed models in which proton pumping is directly coupled to the oxygen reduction cycle at the heme a_3/Cu_B binuclear center of the oxidase (*6*, *7*, *9*−*13*). There are, however, different observations on the number of chemical and pumped protons which are translocated during the oxidative and reductive steps of the catalytic cycle (*6*, *10*, *14*−*16*).

pH dependence of the midpoint redox potential (*17*, *18*) and direct measurements of proton release/uptake by COX[1] observed upon anaerobic oxidation/reduction of the metal centers of cytochrome *c* oxidase in the soluble state show cooperative H^+/e^- linkage in heme *a*, Cu_A (*18*, *19*), heme a_3, and Cu_B (*8*, *20*) (redox Bohr effects (*3*)). Cooperative linkage between the redox state of Fe_{a3} and Cu_B (and catalytic intermediates of oxygen reduction) and p*K*'s of propionate

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substituents of the heme porphyrin and nearby residues is conceived to be involved in the pump mechanisms confined to the binuclear center (for review, see ref *11*). Models have also been proposed with a key role in the translocation of pumped protons of cooperative H^+/e^- linkage at heme *a* (and Cu_A) (*6*, *15*, *21*−*27*).

X-ray crystallographic analysis of heme-copper oxidases (*28*−*31*) and mutational analysis (*5*, *12*, *32*) show pathways that can conduct protons from the N aqueous space to the redox centers, buried in the protein at discreet distances from the membrane surfaces. Less is known on the proton output pathway to the P aqueous space (*28*, *29*−*33*) in which structured water molecules can be involved (*34*).

A detailed study is presented here on the stoichiometry of H^+ release from purified bovine-heart citochrome *c* oxidase reconstituted in phospholipid vesicles (COV) in the oxidative (R → O transition), reductive phase (O → R transition), and in the overall catalytic cycle of the oxidase (R → O → R transition). It is directly shown that protons are released from COV in the external P phase both in the R → O transition and in the O → R transition. The H^+/COX ratios for proton release in these transitions, as well as in the R → O → R transition, are found to exhibit a peculiar pH dependence. The results show involvement in the proton pump of H^+/e^- cooperative coupling at the metal centers (redox Bohr effects) and protonmotive steps of the reduction of O_2 to H_2O.

MATERIALS AND METHODS

Materials. Horse heart cytochrome *c* (type VI), valinomycin, riboflavin, phospholipid (L-α-lecithin, L-α-phosphatidylcholine, type II-S, from soybean), catalase, and superoxide dismutase were from Sigma Chemical Co.; hexammineruthenium (II) chloride was from Sigma-Aldrich and potassium ferricyanide from BDH Chemicals, Ltd. All other reagents were of the highest purity grade commercially available.

Enzyme Preparation and Reconstitution in Liposomes. Cytochrome *c* oxidase was purified from beef heart mitochondria as described in ref *35*. The nanomoles of heme $a + a_3$/mg protein were about 10, and SDS−PAGE analysis revealed the complete set of 13 subunits (*36*). The activity of the enzyme preparation measured polarographically in 40 mM KCl, 10 mM Hepes, pH 7.4, 0.1 mM EDTA, 50 μM cytochrome *c*, and 40 nM aa_3, supplemented with 25 mM ascorbate was, at room temperature, around 80 O_2 molecules·s^{-1}·aa_3^{-1}. The oxidase vesicles, 1 mg of protein COX mixed with 40 mg of sonicated phospholipids, were sealed by the cholate dialysis method (*4*, *37*) either in 10 mM K-Hepes (low buffer COV) or a mixture of 34 mM Hepes, 31 mM Tris, and 35 mM MES (high buffer COV) at the pHs specified in the figures. The buffer capacity of this mixture (β) was 25 mM H^+/ΔpH unit in the pH range from 6.5 to 8.5. The respiratory control ratio (rate of electron flow in the presence of valinomycin plus CCCP/rate of electron in their absence), measured polarographically (*4*), was never lower than 15. The right-side-out orientation of the oxidase in liposomes was never below 85% (*4*, *37*).

Measurement of pH and Redox Changes. Soluble cytochrome *c* oxidase (COX) and liposome-reconstituted oxidase (COV) were suspended at a final concentration of 1−1.5 μM aa_3 in 150 mM KCl. The COX or COV suspension was vigorously stirred in a spectrophotometric-thermostated quartz cuvette with a square cross section of 1 cm^2 using a glass-covered magnetic bar. The top of the cuvette was sealed with a stainless steel stopper which had a port for the pH electrode, two small holes for injection needles, and a port for the entry of argon. Residual oxygen was removed from the argon by passing the gas through oxygen-trapping columns (R&D Separation, OT 3-4 and LIOT 4). The gas path was made of glass or stainless steel narrow tubings connected by Teflon Swagelok ferrules. To minimize evaporation of the sample, argon was passed through two successive water-filled bubblers. Injections of H_2O were made using Gastight syringes (Hamilton) driven by a computer-controlled titration-injection pump (Oroboros Instruments). The reaction medium was made almost completely anaerobic in the cell of an oxygraph, where the oxygen concentration was checked potentiometrically, before being introduced in the measuring cuvette under the argon flux.

A simultaneous recording of absorbance and pH changes was carried out with a diode-array spectrophotometer (settled in the multiwavelength mode) and a combined pH electrode with accuracy of 5×10^{-4} absorbance and 10^{-3} pH unit, respectively (*38*). The wavelengths selected were 550−540 nm for cytochrome *c* and 445−470 and 605−630 nm for heme a_3 and heme *a*. The concentration of aa_3 was determined using a micromolar extinction coefficient at 445−470 nm of 0.188 cm^{-1} for heme aa_3 (*39*). This coefficient is higher than the micromolar extinction coefficient at 445−470 nm of 0.152 cm^{-1} (*40*) used in our previous calculations (*15*, *20*). Ferricyanide concentration was determined at 420−500 nm using a $\Delta\epsilon$ of 1.0 mM^{-1}.

Statistical Analysis. The experimental data were analyzed by two-tailed Student's *t*-test.

RESULTS

Proton translocation in purified bovine heart cytochrome *c* oxidase (COX) incorporated in liposomes (COV) was analyzed by measuring pH changes in the external bulk phase in the following transitons: (i) oxidation of the fully reduced COX by a substoichiometric amount of O_2 (Figure 1A); (ii) oxidation directly followed by re-reduction (one complete turnover, Figure 1B); (iii) direct reduction of the fully oxidized COX (Figure 1C). The results of the measurements presented in Figure 1 and summarized in Table 1 show that oxidation by O_2 of the fully reduced oxidase (R → O transition) resulted, in low buffer COV, in the release of 1.91 ± 0.09 H^+/COX, at pH 7.2. This proton release increased slightly in high buffer COV. Anaerobic oxidation of the fully reduced oxidase by ferricyanide resulted, at pH 7.2, in the release of 1.69 ± 0.09 H^+/COX, both in low and high buffer COV (experimental traces not shown). When the oxidation by O_2 of the reduced oxidase was directly followed by full re-reduction of the metal centers, supported by 1.5 μM cytochrome *c* plus an excess of hexaammineruthenium

[1] Abbreviations: COX, purified cytochrome *c* oxidase; COV, cytochrome *c* oxidase reconstituted in phospholipid vesicles; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)-aminometane; Mes, 2-morpholinoethanesulfonic acid; EDTA, ethylendiaminetetracetic acid; EAR (II), hexammineruthenium (II) chloride; FIC, potassium ferricyanide; CCCP, carbonyl cyanide 3-chloro-phenylhydrazone; R, fully reduced cytochrome *c* oxidase; O, fully oxidized cytochrome *c* oxidase.

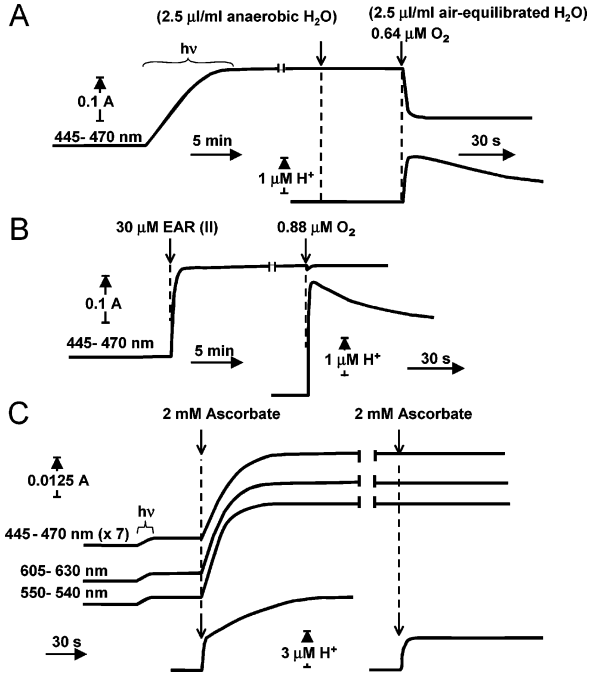

FIGURE 1: Measurement of proton release and redox changes of aa_3 in different phases of the catalytic cycle of cytochrome *c* oxidase in COV. (A) Oxidation by O_2 of the fully reduced oxidase (R → O transition); (B) aerobic oxidation directly followed by re-reduction of the oxidase (R → O → R transition); (C) anaerobic reduction of the fully oxidized oxidase (O → R transition). COVs (high buffer COV), 1 μM aa_3 sealed in the high buffer medium, were suspended in 150 mM KCl supplemented with 4 μM valinomycin, pH 7.2, at 25 °C. The pH changes associated with redox transitions of the metal centers in COX were calibrated with anaerobic-titrated HCl solution. In panel A, the anaerobic reduction of cytochrome *c* oxidase was attained by the photoactivated EDTA/riboflavin system, in the presence of 2 mM EDTA, 0.1 μM riboflavin (*41*), and 0.05 μM cytochrome *c*, with repetitive flashes of actinic light (100 ms, 3000 Lumens). SOD (30 μg) and catalase (3000 U) were also added to 1 mL of the suspending medium to remove oxygen-free radicals eventually produced by direct reduction of oxygen by riboflavin during flashes. No further electron delivery to the oxidase takes place in the absence of flashes (*41*). The fully reduced oxidase was oxidized by the addition of few microliters of air-equilibrated water containing a number of O_2 electron equivalents slightly substoichiometric with those of the four redox centers of the oxidase (the addition of an equivalent volume of anaerobic water is also indicated showing the absence of artifacts). The H^+/COX ratios were obtained by dividing the amount of proton release by that of the COX oxidized (see Table 1). The latter was estimated using a micromolar extinction coefficient of 0.188 cm^{-1} for heme aa_3 at 445−470 nm (*39*). In panel B, the anaerobic reduction of cytochrome *c* oxidase, supplemented with 1.5 μM cytochrome *c*, was attained with the addition of an excess of hexaamminerutenium (II), and the oxidation, re-reduction cycle was produced by the addition of an amount of air-equilibrated water containing a number of O_2 electron equivalents slightly substoichiometric with those of the four redox centers of the oxidase. The H^+/COX ratio was obtained by dividing the protons released by the amount of O_2 added (see Table 1). In panel C, after photoreduction in anaerobiosis of a small amount of the oxidase with the EDTA/riboflavin system (the absence of O_2 in the sample was verified by the absence of COX reoxidation), the reduction of cytochrome *c* oxidase, supplemented with 1.5 μM cytochrome *c*, was produced by 2 mM anaerobic ascorbate. Upon achievement of full reduction of COX, a second pulse of ascorbate was made. The H^+/COX ratio was obtained by subtracting from the pH change associated with the first ascorbate pulse, the artifactual pH change observed upon the second ascorbate pulse. The H^+ release thus calculated was corrected for the scalar H^+ release directly arising from the oxidation of ascorbate to dehydroascorbate equal to 0.5 × Σ reduced equivalents (COX metal centers plus cytochrome *c* reduced).

Table 1

(A)[a]

exp. condition					
R → O	COX_{red}	COX_{ox}	H^+_{rel}		H^+_{rel}/COX_{ox}
	0.95	0.61	1.33		2.18
R → O → R	COX_{red}	$O_{2,\ added}$	H^+_{rel}		H^+_{rel}/O_2
	0.99	0.88	3.10		3.52
O → R	[a]COX_{red}	[b]cyt.c_{red}	[c]H^+_{rel}	[d]$H^+_{ascorbate}$ (4a + b)/2	H^+/COX (c − d)/a
	0.97	1.49	3.23	2.68	0.57

(B)[b]

	low buffer COV			high buffer COV		
exp. condition	H^+/COX	± SEM	*n*	H^+/COX	± SEM	*n*
R → O (O_2)	1.91	0.09	10	2.19*	0.03	3
R → O (FIC)	1.68	0.09	10	1.69*	0.09	3
R → O → R	2.77#	0.08	21	3.66#	0.09	17
O → R	0.14##	0.05	12	0.89##	0.27	3

[a] (A) Experimental data of measurements shown in figure 1. The conditions R → O, R → O → R, and O → R are those of panels A, B, and C of Figure 1, respectively. The values reported for the measured COX, H^+, and cyt.*c* are given as micromolar changes. All the measurements were carried out at pH 7.2 and 25 °C as detailed in the legend of Figure 1. [c]H^+_{rel} is the observed H^+ release, corrected for the artifactual pH change due to the small pH difference between the anaerobic ascorbate solution and the reaction medium. [d]H^+ is the chemical H^+ release due to ascorbate oxidation by electron carriers. [b] (B) Averages of H^+/COX release ratio in different experimental conditions in low and high buffer COV. The condition R → O (FIC) refers to anaerobic oxidation by ferricyanide whose experimental details are described in the legend to Figure 2. All the measurements were carried out at pH 7.2 and 25 °C. *P < 0.01; #P < 0.0001; ##P < 0.0005.

(II) (R → O → R transition), the H^+ release amounted, at pH 7.2, to 2.77 ± 0.08 H^+/COX in low buffer COV and increased to 3.66 ± 0.09 H^+/COX in high buffer COV (P < 0.0001), thus confirming the H^+/COX ratio reported for the R → O → R transition by Wikstrom et al. (*10*, *14*). The present results show that a low internal buffering power of COV and the consequent significant increase of the ΔpH component of the protonmotive force can result in underestimation of the maximal efficiency of proton pumping. This agrees with other observations from our group showing that dissipation of transmembrane ΔpH by low concentrations of the uncoupler CCCP increased the steady-state H^+/e^- ratio of proton pumping in respiring COV (*4*). The lower H^+/COX ratios for proton release from COV in the R → O → R transition previously obtained in our laboratory (*15*, *16*) were due to the low internal buffering power of COV, which were sealed at a low buffer concentration (10 mM K-Hepes), and the use of a smaller micromolar extinction coefficient (at 445−470 nm) than the present one (see Materials and Methods) to determine the aa_3 concentration.

Direct reduction of the oxidase in COV, which was not previously subjected to redox changes (O → R transition), resulted in a proton release which at pH 7.2 amounted to 0.14 ± 0.05 H^+/COX in low buffer COV and reached an H^+/COX ratio of 0.89 ± 0.27 in high buffer COV. This marked effect of the internal buffering power shows that the H^+ release, observed in the O → R transition, does, indeed, involve proton uptake from the inner (N) space (see also Discussion).

In Figures 2 and 3, results of experiments on the effect of pH on proton translocation in the various oxidation/reduction transitions of cytochrome *c* oxidase are presented. In these experiments, high buffer COVs were sealed in the buffer

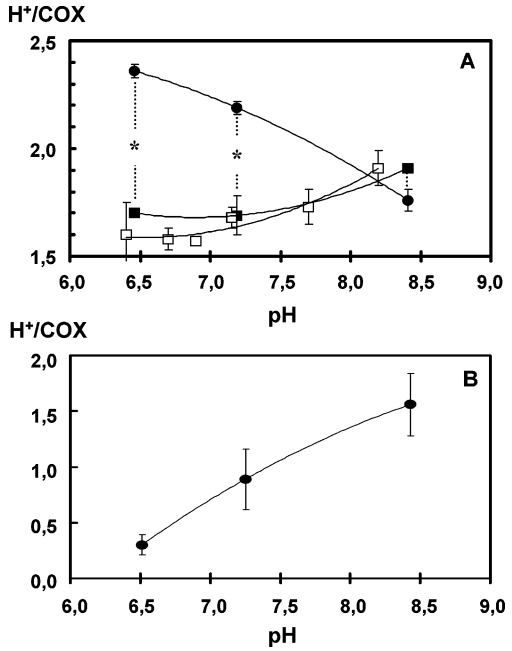

FIGURE 2: pH dependence of proton release associated with the separate oxidation (R → O transition) and reduction (O → R transition) phases of the catalytic cycle of cytochrome *c* oxidase in COV and in the R → O transition of the soluble state. (A) pH dependence of proton release associated with the oxidation of high buffer COV by O_2 (filled circles; $n = 3$, ±SEM) or by anaerobic ferricyanide (filled squares; $n = 3$, ± SEM); pH dependence of proton release associated with oxidation by anaerobic ferricyanide of fully reduced soluble bovine cytochrome *c* oxidase (empty squares). Measurements were carried out as in panel A of Figure 1 for oxidation of COV by O_2. For anaerobic oxidation of COV, the anaerobic reduction (1 μM aa_3 plus 1 μM cytochrome *c*) was attained as in panel A of Figure 1 and the oxidation was produced by addition of an amount of anaerobic ferricyanide stoichiometric with the sum of the reduced metal centers. The H^+/COX ratios reported were calculated dividing the amount of proton release by the amount of COX oxidized (estimated as in panel A of Figure 1). For measurement of the H^+/COX ratio for anaerobic oxidation by ferricyanide of soluble cytochrome *c* oxidase, 1 μM soluble cytochrome *c* oxidase was suspended in 0.15 M KCl, 0.1 mM EDTA and supplemented with 0.3 μM cytochrome *c* and 0.1 mg/mL of frozen-thawed beef heart mitochondria plus 0.5 μM rotenone. The suspension was brought to anaerobiosis, and the metal centers were fully reduced by 5 mM succinate, then 0.1 μM antymicin A and 0.3 μM myxothiazol were added, and oxidation of the metal centers was achieved by adding an amount of anaerobic ferrycianide stoichiometric with the equivalents of the reduced metal centers. Statistical analysis of the difference of the H^+/COX ratios for oxidation by O_2 vs oxidation by ferricyanide of COV at pHs 6.5 and 7.2: *, $P < 0.001$. Difference in the H^+/COX ratios measured upon oxidation by O_2 of COV at pHs 6.46 and 8.41 gave a $P < 0.01$. (B) pH dependence of proton release associated to anaerobic reduction of COV ($n = 3-4$, ± SEM); measurements carried out as in panel C of Figure 1. Statistical analysis of the difference in the H^+/COX ratios measured at pH 6.51 vs pH 8.43 resulted in $P < 0.005$.

mixture (see Materials and Methods) at pHs 6.5, 7.2, and 8.4 and were, respectively, suspended in the measuring medium at the corresponding pH.

The H^+/COX ratio for proton release from COV resulting from oxidation by O_2 of the fully reduced oxidase (R → O transition), amounting to 2.4 ± 0.03 at pH 6.5, progressively decreased as the pH was raised. It was 2.2 ± 0.03 at pH 7.2 and 1.8 ± 0.05 at pH 8.4 (Figure 2A). Analysis of proton release associated with anaerobic oxidation by ferricyanide of the fully reduced oxidase (Figure 2A) revealed two

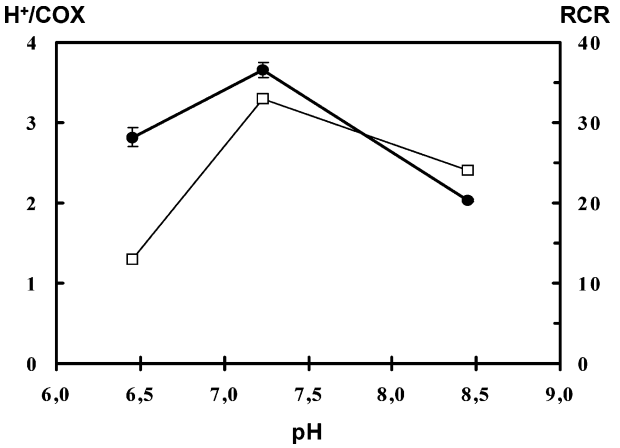

FIGURE 3: pH dependence of proton release associated with the oxidation, rereduction cycle (R → O → R transition) of cytochrome *c* oxidase in COV. Filled circles: H^+/COX ratio ($n = 3-17$; ± SEM) measured as described in panel B of Figure 1. Statistical analysis of the H^+/COX ratio reported at a given pH resulted in a significant difference (P at least < 0.01) when compared with the values reported at the other two pHs. Empty squares: RCR (respiratory control ratio) measured polarographically as in ref *4* (averages of two different COV preparations at each pH).

important features of proton translocation. The H^+/COX ratio for proton release from COV resulting from ferricyanide oxidation of the metal centers of the oxidase amounted only to 1.7−1.9 in the pH range 6.5−8.5; it was thus significantly lower than the H^+/COX ratio measured in the oxidation of COX by O_2 at pHs 6.5 and 7.2. This difference in the H^+/COX ratio observed between the aerobic and anaerobic ferricyanide oxidation of the fully reduced oxidase vanished at pH 8.5 (Figure 2A). Furthermore, the H^+/COX ratio for proton release from COV induced by ferricyanide oxidation was practically the same as the H^+/COX ratio for proton release observed when the fully reduced oxidase in the soluble state was oxidized by ferricyanide (Figure 2A).

Figure 2B shows the pH dependence of proton release from COV resulting from direct reduction of the fully oxidized oxidase, which was not previously subjected to redox changes (O → R transition). In this case, the H^+/COX ratio increased progressively with pH from 0.3 ± 0.09 at pH 6.5 to 0.89 ± 0.27 at pH 7.2 and 1.6 ± 0.28 at pH 8.5.

Figure 3 shows that in the R → O → R transition the H^+/COX ratio for proton release from COV decreased from the value of 3.7 ± 0.09 at pH 7.2 to 2.8 ± 0.11 at pH 6.5 and to 2.0 ± 0.01 at pH 8.5, respectively. Separate controls showed that the index of respiratory control was 33 at pH 7.2 but decreased significantly both at pH 6.5 and pH 8.5.

DISCUSSION

The present study shows that proton pumping in cytochrome *c* oxidase, which at pH 7.2 reaches a maximal efficiency of 4 H^+/COX released from COV in the overall catalytic cycle (R → O → R transition), is distributed in a pH-dependent fashion between the oxidative and reductive phases of the cycle.

A total of 1.7−1.9 H^+/COX of the overall proton release from COV detected in the pH range 6.5−8.5 upon oxidation by O_2 of the fully reduced cytochrome *c* oxidase (R → O transition) originates from deprotonation of acidic groups in the oxidase cooperatively linked to oxido/reduction of the

metal centers of the enzyme (redox Bohr protons; refs *3*, *8*, *20*). This is, in fact, the number of protons released from the oxidase, both when incorporated in COV or in the soluble state, upon oxidation of the fully reduced oxidase by ferricyanide in the absence of O_2 (see Figure 2A). The additional proton release from COV detected when the oxidase is oxidized by O_2, at pHs 6.5 and 7.2, is thus associated with reduction of O_2 by the reduced metal centers of the enzyme. This extra proton release tends to 1 H^+/COX at acidic pH but progressively disappears as the pH is raised to 8.5 (Figure 2A). The H^+/COX ratio for the proton release associated with reduction of the oxidized enzyme increases, on the contrary, as the pH medium is raised from 6.5 to 8.5 (Figure 2B). We note that proton release in the R → O transition derives essentially from deprotonation of acidic clusters linked to the redox centers of COX, close to the outer P side of the membrane (*11*, *34*). This is consistent with the observed small effect of the internal buffering power on H^+ release from COV in this transition (see Table 1). Previous work from our laboratory (*20*) has shown that protonation to H_2O of the two OH^- produced and bound at the binuclear heme a_3−Cu_B center, upon aerobic oxidation of the fully reduced soluble oxidase to the O state, at pH ≈ 6.5, takes place almost completely in the oxidative phase. At pH 8.5, OH^- bound to the binuclear center is not completely protonated to H_2O unless it is released from the center upon its reduction (*20*). X-ray crystallographic data show, in fact, that the space between heme a_3−Fe and Cu_B is occupied by two oxygen atoms in the oxidized state but is apparently empty in the reduced state (*26*). It can be argued that in the membrane-associated oxidase around 1 H^+/COX is taken up from the N space by acidic group(s) (C_2 cluster) in the environment of the binuclear center, upon reduction of O_2 to $2OH^-$. This proton would then be released in the P space upon protonation of OH^- to H_2O by incoming protons from the N space (cf. refs *9*, *42*).

Oxidation/reduction of heme *a* and Cu_A in the soluble carbon monoxide-inhibited cytochrome *c* oxidase has been found to be coupled with release/uptake of 0.7−0.9 H^+/COX in the pH range 6.0−8.5 (*18*, *19*, see also Forte et al. (*43*)). Thus, about one-half of the overall Bohr protons released from the unliganded soluble oxidase or COV upon anaerobic oxidation of the metal centers can derive from deprotonation of the protolytic cluster (C_1) cooperatively linked to oxido-reduction of Cu_A/heme *a*, the other half from the protolytic C_2 cluster linked to heme a_3−Cu_B. Electron/proton coupling at heme *a* only, with a H^+/COX coupling ratio of 0.7−0.9, was apparently inconsistent with previous measurements of the pH dependence of the E_m of heme *a*, which in the CO-inhibited COX was reported to decrease not more than 10−20 mV per pH unit increase in the same pH range (*44*). A solution to this was provided by the finding that Cu_A exhibits the E_m value and a pH dependence completely superimposed on that of heme *a*, the E_m of both centers decreasing by around 20 mV/pH unit increase (*18*). This shows that oxido-reduction of both heme *a* and Cu_A is linked to p*K* shifts of two or more common acid−base groups, whose overall balance results in the observed H^+ release upon oxidation of the two centers and proton uptake upon their reduction (*45*). An implication of the interactive coupling of the oxido-reduction of both Cu_A and heme *a* with p*K* shifts in a common cluster of protolytic groups is that, while one electron reduction of Cu_A/heme *a* is sufficient to produce maximal protonation of the cluster, release of the proton from the cluster will take place only when both heme *a* and Cu_A are oxidized (see Figures 2 and 7 in ref *19*). At the steady-state, one electron at a time has, hence, to pass through Cu_A and heme *a* to result in the translocation of around 1 H^+ per electron (see scheme in Figure 4). This might be one of the causes of the slip in the proton pump of the oxidase observed at high pressure of electron delivery to the enzyme and high transmembrane ΔpH (*4*, *46*, see also *47*).

In the reductive phase of COV, upon transfer of two electrons, one at a time, from ferrocytochrome *c* to heme a_3−Cu_B, via Cu_A−heme *a*, ≈2 H^+/COX will be taken up from the N space by the cluster C_1 linked to Cu_A/heme *a* and then translocated from this to the cluster C_2 linked to heme a_3/Cu_B (the two clusters acting in series can constitute the gate of the pump) (*16*). One-half of these protons are released in the P space, the other protonate the cluster C_2 linked to Cu_B/heme a_3 (*16*). At alkaline pH, reduction of the binuclear center releases OH^- bound at the center, and its protonation to H_2O by incoming H^+ from the N space causes release in the P space of 1 H^+ from the C_2 cluster whose protonation/deprotonation is governed by the reduction state of heme a_3/Cu_B and the OH^-/H_2O couple, (Figure 2B). At alkaline pH, upon full reduction of oxidized COX (O → R transition), up to five protons are taken up from the inner (N) space and move along 65−70% of the membrane-spanning part of COX to the environment of the redox centers close to the P side of the membrane. Two protons are consumed in the production of water at the heme a_3/Cu_B center; up to two H^+'s are released in the outer P phase; the remaining H^+ protonates the acidic clusters cooperatively linked to the redox centers. This is reflected in the marked effect of the internal buffering power on the H^+ translocation in COV observed in this transition (see Table 1). H_2O produced at the binuclear center can favor H^+ release in the P phase by lowering the p*K* of the acidic group(s) linked to the OH^-/H_2O couple at the binuclear center. A critical event for the release of the pumped protons in the outer P space can be represented by the gating mechanism which will ensure unidirectional movement of the product water toward the exit pathway in the external (P) space, which might be shared by the pumped protons on their release in the P space (*34*). The low H^+ release observed in the O → R transition at acidic pHs tending to vanish at pHs ≤ 6 could be due to a slip in the coupling of proton translocation associated with oxido-reduction of Cu_A/heme *a* if, under the electron delivery pressure exerted in these conditions, electron transfer from heme *a* to the binuclear heme a_3−Cu_B is simultaneous with reduction of Cu_A by cytochrome *c* so that Cu_A/heme *a* remain half-reduced (*19*). An additional reason for the low H^+/COX ratio at acidic pHs in the O → R transition could be due to higher effective p*K* of the C_1 cluster in the free unliganded oxidase, as compared to that calculated in CO-liganded oxidase in which both heme a_3 and Cu_B are clamped in the reduced state (*8*). The reasons why Verkhovsky et al. (*14*) failed to observe significant H^+ release upon direct reduction of the oxidized enzyme are not clear. One reason could be the slip of the pump under their experimental conditions and/or complete displacement by chloride of the OH^- bound at the oxidized binuclear site in their oxidase preparation (*43*).

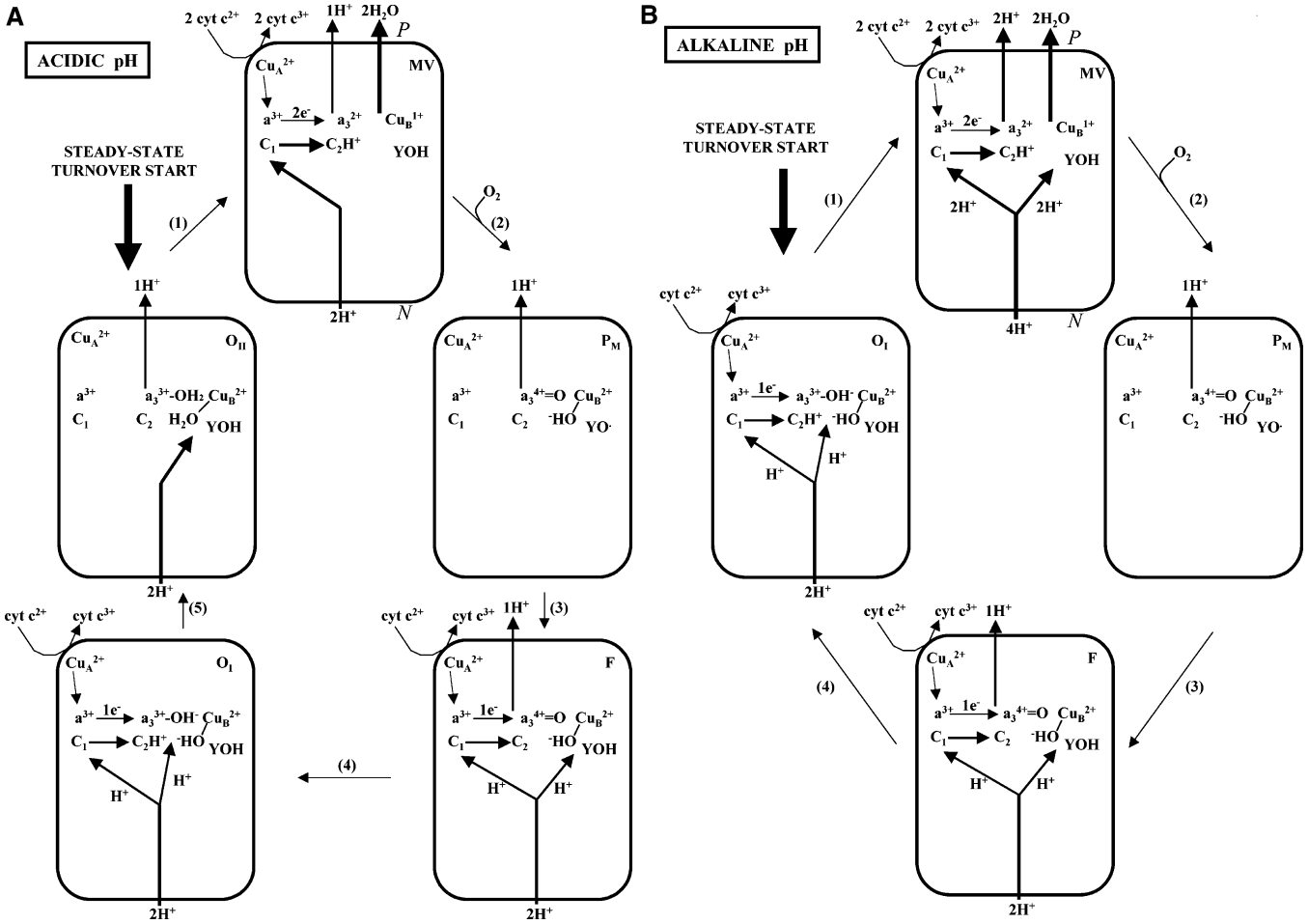

FIGURE 4: Model of the catalytic cycle of reduction of O_2 to H_2O by ferrocytochrome *c* and proton pumping in the cytochrome *c* oxidase in the coupling membrane at the respiratory steady state, at acidic (A) and alkaline (B) pHs. *P*, outer aqueous space; *N*, inner aqueous space. The steady-state turnover of the oxidase starts with electron delivery from ferrocytochrome *c* to the fully oxidized COX. Upon transfer of the first two electrons to the heme a_3/Cu_B center, the MV state is generated (step 1). Molecular oxygen reacting with the MV state is reductively cleaved generating the P_M state (step 2). Transfer of the third electron converts the P_M to the F state (step 3) which is finally converted to the O state upon delivery of the fourth electron (step 4). Proton consumption in the production of the two water molecules at the binuclear center and pumping of the four protons are differently distributed at acidic and alkaline pHs among the sequential steps of the reductive and oxidative phases of the catalytic cycle of the oxidase.

Time-resolved spectroscopic analysis, mutational analysis of bacterial cytochrome *c* oxidases, and X-ray crystallography structures have provided detailed characterization of chemical intermediates in the reduction of oxygen to water and their relationship with proton translocation (for review, see refs *5*, *11*, *48*). In Figure 4, a mechanism is presented which attempts to integrate the present observations with the intermediate steps in the catalytic cycle of cytochrome *c* oxidase at the respiring steady state. The mechanism, derived from what was observed with the bovine enzyme, can equally be applied to bacterial cytochrome *c* oxidases. For the sake of clarity, the cases of acidic and alkaline pHs are presented separately.

A steady-state turnover of the oxidase starts with the fully oxidized enzyme. At acidic pH, two water molecules produced in the final step of the oxidative phase of the cycle are bound to heme a_3/Cu_B. Transfer of the first two electrons from ferrocytochrome *c* to heme a_3/Cu_B, one at a time, via Cu_A/heme *a* (generation of Mixed Valence state), results in the release of the two water molecules in the external P space. Two H^+'s are taken up from the inner N space. One H^+ is held by the cluster C_2 in reduced state of Fe_{a3}/Cu_B; the other H^+ is pumped in the P space.

O_2 arriving at the reduced binuclear center undergoes a $4e^-$ reductive cleavage, two electrons come from the oxidation of Fe_{a3}^{2+} to Fe_{a3}^{4+}, one from Cu_B^{1+}, and the fourth, together with a proton, from a Tyr residue (I-Y244, in the bovine enzyme), with generation of the P_M intermediate (*11*, *48*). The oxidation of the binuclear center results in the release in the P space of the second pumped proton, which was held by the C_2 cluster which can be located quite close to the P side (cf. reviews in refs *11*, *34*). No chemical protons are taken up from the N space in the MV−P_M transition (*49*). Thus, no significant electrogenic event can be observed in this transition (cf. ref *50*). The transfer of the third electron via Cu_A/heme *a* to the binuclear site, with conversion of the P_M to the F intermediate, results in the pumping of a third proton from the N to the P space (the pumped proton is translocated via C_1 and C_2 acting in series). The Tyr radical is reduced with the uptake of the first chemical proton from the N space. The transfer of the fourth electron via Cu_A/heme *a* to the binuclear center (conversion of F to O_I) is associated with the uptake of 2 H^+'s from the N space. One is the second chemical proton utilized in the conversion of Fe_{a3}^{4+}═O to Fe_{a3}^{3+}—OH; the other is the fourth pumped H^+. We propose here that this pumped H^+, taken up from the N

space always via C_1, is transferred to the C_2 cluster and held there transitorily by the presence of the OH^- bound to Fe_{a3}^{3+}. In a final step, the third and the fourth chemical protons are taken up from the N space and, at acidic pH, protonate the two OH^- to 2 H_2O. Water formation at the binuclear center lowers the effective p*K* of C_2 and promotes the transfer of the pumped proton to the P space (O_{II} state).

At alkaline pH, the proton pumping steps in the conversions of MV to P_M, P_M to F, and F to O_I are similar to those taking place at acidic pH, but the protonation of OH^-, bound to heme a_3/Cu_B, to water does not take place until it is released from the binuclear center upon reduction. Thus, at alkaline pH, four protons are taken up from the N space upon reduction of the O_I intermediate to the MV intermediate. Two of the four protons are pumped in the P space (the first and the fourth in the cycle at acidic pH) together with the two water molecules formed upon protonation of the two OH^-'s by the other two incoming H^+'s from the N space. At physiological pHs, the contribution of the reductive and oxidative phase to proton pumping, in particular that of steps 1 and 5, will be intermediate between the extreme cases described in Figure 4 for acidic and alkaline pHs. The pH dependence of the H^+/COX ratio for proton translocation in the R → O → R transition and of the index of respiratory control indicates that the highest efficiency of the proton pump is reached at pH 7.2. Apparently, at this physiological pH, optimal conditions are met for the partition of the work for proton pumping between the oxidative and the reductive phases of the catalytic cycle of the oxidase.

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