Factors Affecting the H^+/e^- Stoichiometry in Mitochondrial Cytochrome *c* Oxidase: Influence of the Rate of Electron Flow and Transmembrane ΔpH[†]

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ABSTRACT: A study is presented of the factors affecting the H^+/e^- stoichiometry of the proton pump of mitochondrial cytochrome *c* oxidase, isolated and reconstituted in phospholipid vesicles (COV). Under level flow conditions, i.e., in the absence of a transmembrane $\Delta\mu H^+$, the H^+/e^- ratio, obtained from spectrophotometric measurements of the initial rates of electron flow and H^+ release specifically elicited by cytochrome *c*, varied from around 0 to 1, depending on the actual rate of electron flow through the oxidase. At steady state the H^+/e^- ratio for the oxidase was specifically depressed by the transmembrane ΔpH. The study of the H^+/e^- ratio of the pump was complemented by an analysis of the redox pattern of cytochrome *c*, Cu_A, and heme a. From both sets of results and recent structural data from other groups, it is concluded that the dependence of the H^+/e^- ratio on the rate of electron flow through the oxidase and transmembrane ΔpH is associated with the possible occurrence of two electron transfer pathways in cytochrome *c* oxidase, a coupled one (cyt *c* → Cu_A → heme a → heme a_3-Cu_B) and a decoupled one (cyt *c* → Cu_A → heme a_3-Cu_B). The contributions of the two pathways, differently affected by kinetics and thermodynamic factors, will determine the actual H^+/e^- ratio of the pump. A possible role of heme a in the proton pump and the physiological implication of the variable H^+/e^- ratio in the oxidase are discussed.

Cytochrome *c* oxidase (E.C. 1.9.3.1), the terminal complex of the mitochondrial respiratory chain, belongs to the superfamily of protonmotive heme−copper oxidases (Saraste, 1990; Calhoun et al., 1994). These enzymes catalyze the transfer of electrons from cytochrome *c* or quinol (the latter in some prokaryotic oxidases) to dioxygen. Electron flow in heme−copper oxidases results in the generation of an electrochemical proton gradient, Δp[1] (Mitchell, 1966; Papa 1988; Babcock & Wikström, 1992). This derives, in the first place, *directly* from the membrane anisotropy of reduction of O_2 to H_2O, whereby electrons are donated by cytochrome *c* (or quinol) at the P side of the membrane and protons are taken up from the N aqueous phase. In addition, electron flow in protonmotive oxidases is associated with proton pumping from the N to the P aqueous phase (Wikström et al., 1981).

Direct (Mitchell, 1987) and indirect models (Malström, 1985; Gelles et al., 1986) have been proposed to explain the proton pump activity of heme−copper oxidases (Papa et al., 1994). Important progress toward elucidation of the mechanism of the pump has recently been made with mutational analysis of prokaryotic oxidases (Hosler et al., 1993; Calhoun et al., 1994; Villani et al., 1995) and now with the determination by X-ray crystallography at 2.8-Å resolution of the structure of cytochrome *c* oxidase, in particular subunit I, in *Paracoccus denitrificans* (Iwata et al., 1995) and bovine heart (Tsukihara et al., 1995). Subunit I, which is highly conserved in protonmotive oxidases, contains the heme−copper center where O_2 is reduced to H_2O and proton pumping is generally thought to be coupled to redox catalysis (Mitchell, 1988; Malmström, 1990; Babcock & Wikström, 1992; Rousseau et al., 1993; Woodruff, 1993; Papa et al., 1994).

Direct models based on proton conduction by the same oxygen-reduction intermediates (Mitchell, 1987) predict, in principle, a fixed overall stoichiometry of 1 H^+/e^-. This stoichiometry can, however, decrease if a fraction of the electrons flows through a decoupled redox pathway. Indirect models, based on cooperative (Papa, 1976) and/or kinetic linkage (Blair et al., 1986; Malmström, 1985, 1989) between electron transfer at the metals and proton transfer by protonable groups in the enzyme, can produce variable H^+/e^- stoichiometries due to electron and/or proton slips (Pietrobon et al., 1983; Papa, 1988; Papa et al., 1994; Musser & Chan, 1995).

The H^+/e^- stoichiometry in cytochrome *c* oxidase is studied with indirect and direct approaches. In the first, the steady-state relationship between respiratory rate and Δp is analyzed under a variety of conditions (Murphy, 1989). The observed nonlinearity of this relationship (Nicholls, 1974; Pietrobon et al., 1983; Brown & Brand, 1986) is taken by some authors as evidence of a slip in the proton pump (Pietrobon et al., 1983; Luvisetto et al., 1991) and by others as due to nonohmic increase of membrane proton conductance at high Δp (leak) (Brown & Brand, 1986; Brown, 1989). The H^+/e^- stoichiometry of proton pumping is measured directly with two methods: the oxygen or reductant pulse (Mitchell et al., 1979) and the rate method (Reynafarie et al., 1979; Papa et al., 1973; Murphy & Brand, 1988). Papa et al. (1991), using the latter, have shown that the intrinsic H^+/e^- stoichiometry of cytochrome *c* oxidase, both in

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[1] COX, cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, E.C. 1.9.3.1); COV, cytochrome *c* oxidase vesicles; ΔpH, transmembrane pH gradient; Δp, transmembrane electrochemical proton gradient; v_i^{e-}, equivalents of electrons aa_3^{-1} s^{-1}; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazome; EDTA, ethylenediaminetetracetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SEM, standard error mean.

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mitochondria and in the isolated reconstituted state (Capitanio et al., 1991), varies as a function of the rate of electron flow.

In this paper, a study is presented of the influence of the rate of e^- flow and the ΔpH component of Δp on the H^+/e^- stoichiometry of proton pumping by isolated bovine heart cytochrome *c* oxidase reconstituted in phospholipid vesicles. Under level flow conditions, the H^+/e^- ratio is markedly influenced by kinetic factors which can vary the stoichiometry from 1 to close to 0. Under steady-state conditions, transmembrane ΔpH causes a decrease of the H^+/e^- ratio well below 1, which results from intrinsic decoupling of the pump. A discussion of the mechanistic and physiological implications of these observations is presented.

MATERIALS AND METHODS

Enzyme Preparation and Reconstitution in Liposomes. Cytochrome *c* oxidase was purified from bovine heart mitochondria according to Errede et al. (1978); the number of nanomoles of heme $a+a_3$/milligram of protein was 9−10 and the SDS−PAGE analysis, performed as in Kadenbach et al. (1983), revealed the presence of all 13 subunits in the purified complex. Cytochrome *c* reductase was purified from bovine heart according to Rieske (1967).

Preparation of Cytochrome c Oxidase Vesicles. Reconstitution of cytochrome *c* oxidase in phospholipid vesicles (COV) was performed by the cholate dialysis method as described by Casey et al. (1979). Preparation of COV with entrapped pyranine was performed as follows: 5 mM pyranine was added to the sonicated detergent/phospholipid/protein mixture and was also present, at the same concentration, in the medium of the first dialysis step; the composition of the dialysis medium was 1 mM K-Hepes, pH 7.2, 30 mM KCl, and 100 mM sucrose. The dialyzed vesicles were passed through a Sephadex G-25 column (equilibration and elution were made with the basic assay medium, see below). This procedure allowed for extensive removal of the external pH probe without affecting the respiratory control ratio in COV (never below 10, when measured polarographically; Papa et al., 1987) or the asymmetric right-side-out orientation of the oxidase molecules in the liposomal membrane (never below 85%; Casey et al., 1981).

Measurement of Proton Translocation in COV. Redox-linked proton translocation under "level flow" conditions (see text) was measured according to the method described in Papa et al. (1980, 1991) and Capitanio et al. (1991). The initial rates of pH changes and O_2 consumption, elicited upon activation of the electron flow in an aerobic COV suspension, were estimated spectrophotometrically from the absorbance changes of the phenol red pH indicator (558−593 nm) and deoxygenation of oxyhemoglobin (577−568 nm, $\Delta\epsilon = 6.3$ mM^{-1} cm^{-1}), respectively. Measurement of proton translocation under steady-state conditions was performed potentiometrically, simultaneously following pH changes by a fast-responding combined pH electrode and O_2 consumption by a Clark-type electrode coated with a high-sensitivity membrane.

Fluorescence and Absorbance Measurements. Intraliposomal pH changes attained under steady-state conditions were determined by following the fluorescence of the entrapped pyranine pH indicator (excitation at 460 nm, emission at 520 nm); the external pH was simultaneously monitored by a pH electrode. The actual internal pH was estimated under fully uncoupled conditions by back-titration of the fluorescence quenching with 0.1 M KOH. Redox changes of cytochrome *c*, heme a, and Cu_A were followed at 550−540 nm, 605−630 nm, and 825−740 nm, respectively, by a dual-wavelength spectrophotometer. Membrane potential in respiring oxidase vesicles was followed spectrophotometrically at 526−560 nm using the "membrane potential indicator" safranine O as described in Singh and Nicholls (1985).

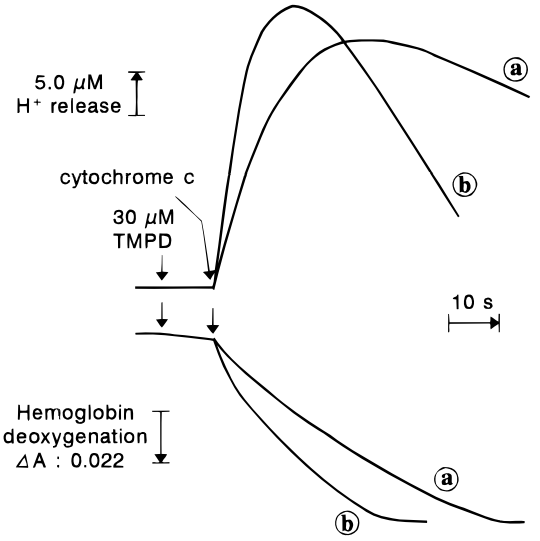

FIGURE 1: Measurement of redox-linked proton translocation in cytochrome *c* oxidase vesicles under level flow conditions. Cytochrome *c* oxidase vesicles (0.35 μM aa_3 final concentration) were suspended in a buffer containing 100 mM Choline-C1, 0.1 mM EDTA, 2.0 μg of valinomycin/mL, pH 7.4. For pH measurements (upper traces) the medium was supplemented with 50 μM phenol red and the absorbance changes were calibrated with standard solutions of 10 mM HCl and KOH; for O_2 consumption measurements (lower traces) the medium was supplemented with 30 μM purified human hemoglobin [factor *f* for the preparation used was 2.1; see Papa et al. (1980)]. The oxygen concentration was lowered by a stream of argon until absorbance changes at 577−568 nm showed that hemoglobin was 50% deoxygenated [for details of the procedure see Papa et al. (1980) and Capitanio et al. (1991)]. The reaction was started by pulsing 0.5 μM (traces a) or 1.5 μM (traces b) ferricytochrome *c* in the presence of 44 mM ascorbate plus 30 μM TMPD. The H^+/e^- ratio measured from the initial rates of proton release and O_2 uptake were 1.28 and 0.79 for experiment a and b, respectively; correction for the scalar proton release associated with the oxidation of ascorbate (i.e., 0.5 H^+/e^-) gave values for the vectorial proton translocation of 0.78 and 0.29.

Chemicals. Horse heart cytochrome *c* (type VI), soybean phospholipids (type II), antimycin A, valinomycin, nigericin, CCCP, and safranine O were from Sigma Chemical Co.; duroquinol was from K. & K. Laboratories; pyranine was from Eastman Kodak Co.; TMPD was from BDH Chemical Ltd. All other reagents were of the highest purity grade commercially available.

RESULTS

Level Flow H^+/e^- Ratio. Figure 1 illustrates the measurement, under level flow conditions, of the H^+/e^- ratio for proton pumping by purified bovine heart cytochrome *c* oxidase reconstituted in liposomes (COV). H^+ translocation and respiration were measured by following optical changes of the phenol red pH indicator and of hemoglobin. COV suspension was supplemented with ascorbate plus TMPD, and cytochrome *c* oxidase activity was elicited by the addition of cytochrome *c*. Separate titrations were carried

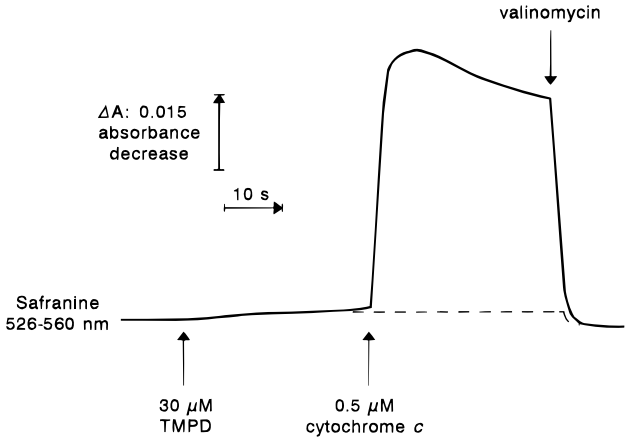

FIGURE 2: Respiration-induced transmembrane potential in cytochrome *c* oxidase vesicles. Safranine (20 μM) was used to monitor spectrophotometrically the generation of membrane potential in the COV suspension (Singh & Nicholls, 1985). The experimental conditions are those of Figure 1 (trace a), with the only difference that 2 μg of valinomycin/mL was omitted from the assay medium and was added when the membrane potential was fully developed upon addition of cytochrome *c* (full trace); the dashed trace refers to the COV supplemented with ascorbate and TMPD alone.

out to find optimal concentrations of ascorbate and TMPD which, without giving *per se* significant oxygen uptake, supported sustained oxidase activity in the presence of cytochrome *c*. Ascorbate (44 mM) plus 30 μM TMPD underwent very slow autooxidation with oxygen uptake amounting to not more than 0.1−0.5% of the respiratory activity of cytochrome *c* oxidase. When these amounts of ascorbate and TMPD were added to COV, in the absence of cytochrome *c*, no appreciable enhancement of the rate of oxygen uptake was observed as compared to their autooxidation, neither H^+ release nor generation of membrane potential of the same sign as that produced upon addition of cytochrome *c* was detected (Figure 2). Proton pumping by cytochrome *c* oxidase was measured in the presence of valinomycin plus K^+, which collapses membrane potential (Figure 2). The H^+/e^- ratio, obtained from the initial rates of H^+ release and O_2 consumption elicited by addition of ferricytochrome *c* to COV, amounted to 0.8 (after correction for the chemical protons arising from oxidation of ascorbate to dehydroascorbate) upon addition of 0.5 μM cytochrome *c* but was only 0.3 with 1.5 μM cytochrome *c*, which gave a higher respiratory rate.

Figure 3 presents the results of a systematic analysis of the dependence of the H^+/e^- ratio on the respiratory rate in COV. The respiratory rate was varied by changing the concentration of cytochrome *c* which was added to COV, always supplemented with 44 mM ascorbate and 30 μM TMPD. In previous experiments the respiratory rate of COV was varied by changing the concentration of TMPD, at a constant cytochrome *c* concentration (Capitanio et al., 1991), a condition which could have introduced complications due to some oxidase activity supported directly by higher concentrations of TMPD in the absence of cytochrome *c* (Crison & Nicholls, 1991). The results of the experiments of Figure 3 (curve a) show that the H^+/e^- ratio, which amounted to 0.2 at a low respiratory rate, first increased to around 0.8 as the rate was enhanced by raising the amount of cytochrome *c* and then started to decrease as the rate was further enhanced. A similar rate-dependence pattern for the H^+/e^- ratio was observed when electrons were fed to COV

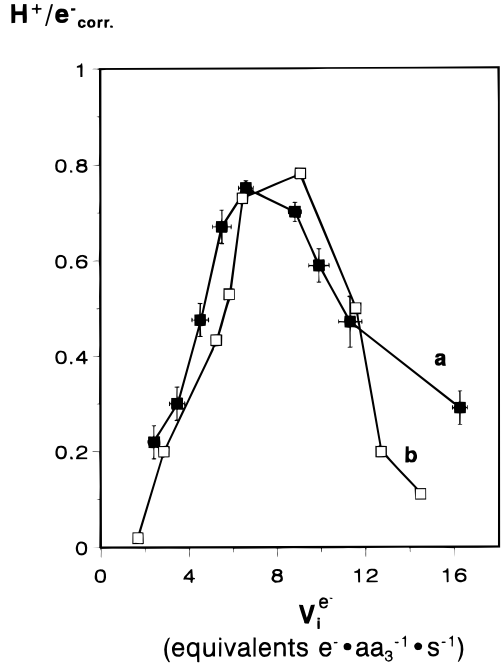

FIGURE 3: Rate dependence of the H^+/e^- ratio for redox linked proton translocation measured in COV under level flow conditions. For trace a (■), the experimental conditions are those described in the legend of Figure 1; the respiratory rate (e^- transferred aa_3^{-1} s^{-1}) was varied, changing the concentration of cytochrome *c* from 0.25 to 1.5 μM. For trace b (□), the electron delivery system to the oxidase vesicles was 200 μM duroquinol, soluble bovine hear purified cytochrome *c* reductase, and 0.5 μM cytochrome *c*. The respiratory rate was modulated, varying the concentration of cytochrome *c* reductase from 0.02 to 0.20 μM. The H^+/e^- ratios were corrected for scalar proton release arising directly from the oxidation of the electron donors that was 0.5 and 1.0 H^+/e^- for ascorbate and duroquinol, respectively. The number of experiments with different COV preparations was 4 (a) and 2 (b). For trace a, the values were clustered and the SEM was reported.

by the soluble bc_1 complex and cytochrome *c* with duroquinol as substrate (Figure 3, curve b). In this latter set of experiments, the rate of electron flow was varied by changing the amount of the bc_1 complex added in a range in which it did not support any respiratory activity in the absence of cytochrome *c*.

Reduction Kinetics of Redox Carriers. Figure 4 shows the time course of reduction of cytochrome *c*, heme a, and Cu_A upon activation of electron flow in COV, supplemented with valinomycin plus K^+, at rates of 7 (traces A) and 16 (traces B) equiv of e^- aa_3^{-1} s^{-1}, giving H^+/e^- ratios of 0.8 and 0.3, respectively (cf. Figure 3). In both cases, all three redox carriers showed an initial rapid reduction which was completed in about 1−2 s and was followed, after a particularly evident lag for Cu_A, by a slower reduction until a steady-state level was reached. The delayed reduction was evidently due to the buildup of ΔpH, as it was completely abolished by CCCP. The uncoupler had, on the other hand, no effect on the initial rapid reduction of the redox carriers, confirming that the initial phase, in which the rates of e^- flow and H^+ translocation were measured to obtain the H^+/e^- ratio, (experiments of Figures 1 and 3), did in fact correspond to a level flow condition. In this condition kinetic factors prevail and no significant effect is exerted by Δp. The initial and steady-state reduction levels of the redox carriers, calculated by means of mathematical deconvolution of their reduction kinetics (Figure 5), are presented in Table 1. A number of points can be noted: (i) At both low and

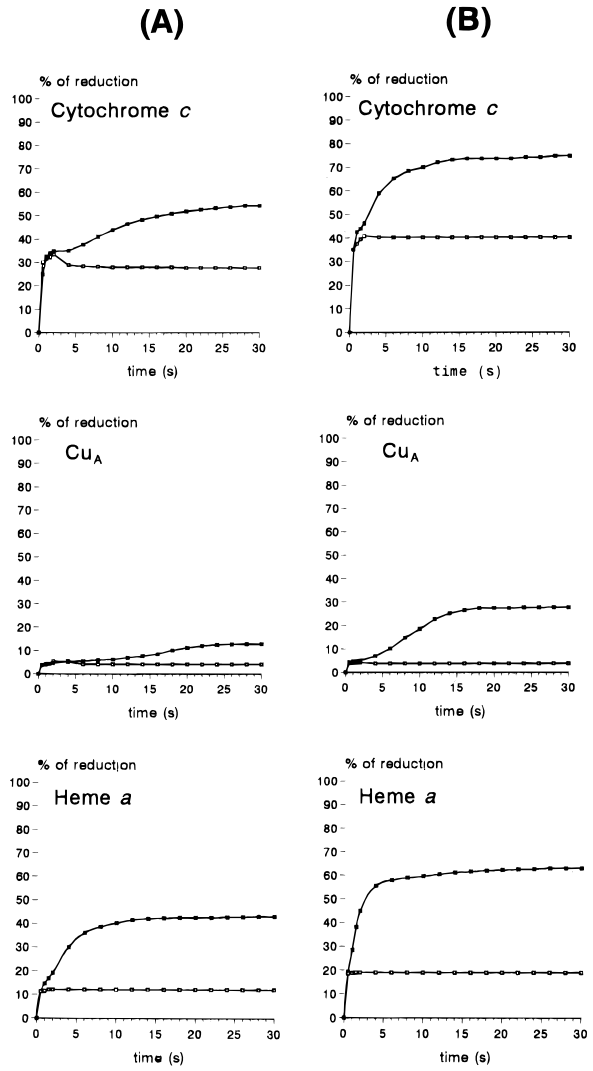

FIGURE 4: Time course of the reduction levels of cytochrome *c*, Cu_A, and heme a. Redox changes were followed spectrophotometrically at the wavelength couples 550−540 nm, 825−740 nm, and 605−630 nm for cytochrome *c*, Cu_A, and heme a, respectively. To reduce the noise to signal ratio, for measurement, in particular of Cu_A absorbance changes ($\Delta\epsilon$ about 1.0 mM^{-1} cm^{-1}), the COV concentration was raised to 2 μM; ascorbate concentration was 40 mM. In panel A the cytochrome *c*/COV ratio was the same as that of Figure 1a and the concentration of TMPD was 80 μM, giving a $v_i^{e^-}$ and H^+/e^- ratio of 7 and 0.8, respectively, comparable to those of Figure 1a. In panel B the cytochrome *c*/COV ratio was the same as that of Figure 1b and the concentration of TMPD was 200 μM, giving a $v_i^{e^-}$ and H^+/e^- ratio of 16 and 0.26, respectively, comparable to those of Figure 1b. The reduction levels are expressed as percentages of the full reduction attained for each redox center after addition of sodium dithionite. The reduction levels of Cu_A were corrected for the spectral contribution of ferricytochrome *c*. Filled squares refer to the reduction levels attained in the presence of valinomycin; empty squares refer to those measured in the presence of valinomycin plus 3.0 μM CCCP. The time courses were best-fitted as illustrated in Figure 5.

high electron pressure exerted on the oxidase, the initial and steady-state reduction levels of redox carriers were in the order cytochrome *c* > heme a > CuA. (ii) At the lower electron pressure and turnover in the oxidase, the reduction level of heme a increased much more significantly than that of Cu_A, going from the pre-steady-state to the steady-state condition. This increase of the reduction levels was abolished by CCCP. These observations indicate that ΔpH exerts

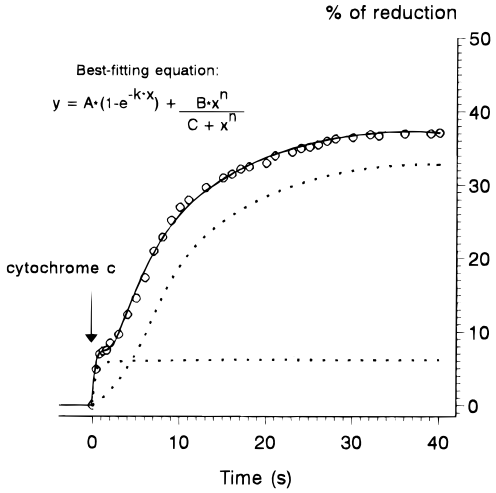

FIGURE 5: Best-fitting procedure for the time courses of the reduction level of heme a in COV under coupled respiration. The figure refers to the reduction levels of heme a (conditions as in Figure 4A) and is representative of the mathematical procedure that was adopted to resolve the kinetic behavior for cytochrome *c* and Cu_A as well. The equation expresses the combined contributions of an initial rapid monoexponential phase and a delayed slower sigmoidal phase (dotted lines), where *y* and *x* are reduction levels and time, respectively, *A* and *k* are the amplitude and rising constant of the exponential phase, and *B* and *C* are the amplitude and $t_{1/2}$ of the slower phase, with *n* being an exponential coefficient introduced to fit its sigmoidal behaviour. Under fully uncoupled conditions the sigmoidal phase was completely abolished and the progress curve of the reduction was, within the experimental variability, comparable with the initial exponential phase resolved from the observed traces under coupled conditions (see Figure 4, Table 1, and text).

a cross-over effect on the oxygen side of heme a (Capitanio et al., 1991; Nicholls & Butko, 1993). (iii) Increase of the electron pressure on the oxidase significantly enhanced the initial reduction level of heme a but had only a minor effect on that of Cu_A. The contrary was observed for the steady-state reduction levels.

Steady-State H^+/e^- Ratios. Figure 6 shows an experiment in which respiration in valinomycin-supplemented COV was sustained by duroquinol with soluble bc_1 and cytochrome *c* until a steady state was reached for redox-linked acidification of the medium and alkalinization for the interior of the vesicles. At this point electron flow was blocked by antimycin, which resulted in immediate backflow of protons from the exterior to the interior of the vesicles. At the steady state the rate of H^+ efflux is equal to that of H^+ influx; the latter is obtained by measurement of the initial rate of the antimycin-induced H^+ influx (Papa et al., 1970; Al Shawi & Brand, 1981). The initial rate of H^+ influx, divided by the steady-state respiratory rate attained before the addition of antimycin, gave the H^+/e^- ratio for proton pumping amounting, after correction for the scalar protons arising from oxidation of duroquinol to duroquinone, to around 0.

In Figure 7, a titration of the effect of small concentrations of CCCP on the steady-state redox-linked proton translocation in COV is presented in a set of experiments like those illustrated in Figure 6. The progressive increase in the proton leak and the decrease of the steady-state respiratory ΔpH, caused by increasing concentrations of CCCP in the nanomolar range, was associated with an increase of the steady-state H^+/e^- ratio for proton pumping.

Table 1: Reduction Levels of Cytochrome *c*, Cu_A, and Heme a Measured in Cytochrome *c* Oxidase Vesicles under Level Flow (Pre-Steady-State) and Steady-State Conditions[a]

	low electron pressure (% reduction)			high electron pressure (% reduction)		
	pre-steady-state	steady-state	+ CCCP	pre-steady-state	steady-state	+ CCCP
cytochrome *c*	20.8 ± 3.4 (7)	53.8 ± 2.7 (7)	20.5 ± 2.9 (4)	29.6 ± 3.8 (6)	70.9 ± 3.6 (6)	31.6 ± 4.3 (4)
Cu_A	6.0 ± 1.3 (5)	14.3 ± 3.1 (5)	5.9 (2)	7.5 ± 2.6 (3)	32.8 ± 2.6 (3)	7.7 (2)
heme a	10.3 ± 2.7 (6)	38.9 ± 6.2 (6)	10.5 ± 0.6 (3)	20.5 ± 3.4 (6)	54.4 ± 5.6 (6)	21.8 ± 1.4 (3)

[a] For experimental details see the legends of Figures 4 and 5. The values are means ± SEM; in parentheses is the number of different COV preparations.

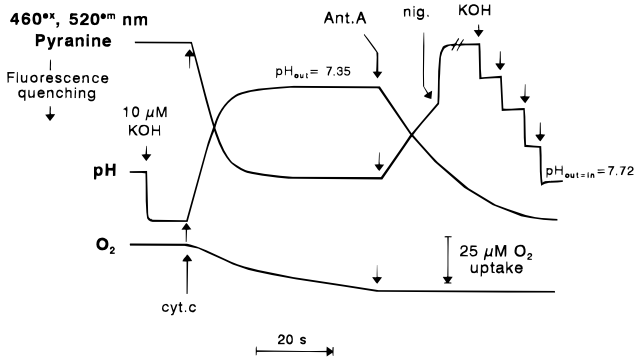

FIGURE 6: Measurements of redox-linked proton translocation and ΔpH generation in COV under steady-state conditions. COV (0.5 μM) loaded with pyranine were suspended in the basic assay medium described in the legend of Figure 1 supplemented with 200 μM duroquinol and 0.125 μM soluble cytochrome *c* reductase. pH was adjusted to 7.4. An aliquot of the suspension was used to measure simultaneously external pH changes and O_2 uptake as described in Materials and Methods. The residual volume was used to measure intraliposomal pH changes, following the fluorescence of the pyranine pH indicator. Electron flow was activated by pulsing 0.5 μM ferricytochrome *c* and, when the stationary condition was attained, was blocked with 0.2 μM of antimycin A. The H^+/e^- ratio was estimated from the initial rate of proton reequilibration following the addition of antimycin A and from the rate of O_2 consumption in the stationary phase (see text). In the experiment reported in the figure, the measured H^+/e^- ratio was 0.89. Control experiments in which the external pH was followed spectrophotometrically by phenol red or the concentration of antimycin A was 0.3 μM gave the same H^+/e^- ratios. This showed that the electrometric approach was not, under these conditions, rate-limiting and that the concentration of antimycin A used did not affect by itself the proton leak across the liposomal membrane. For further details see Material and Methods.

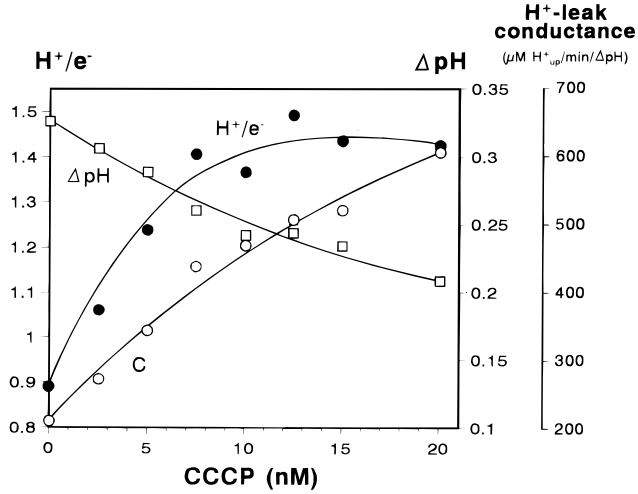

FIGURE 7: Effect of CCCP on proton leak, ΔpH, and H^+/e^- ratios measured under steady-state conditions. Measurements of H^+/e^- ratios (●) and membrane proton-leak conductance (○) were measured spectrophotometrically. Transmembrane ΔpH (□) was measured following pyranine fluorescence. For other details see legend to Figure 6.

DISCUSSION

The results of the present rate-method analysis show that the intrinsic H^+/e^- stoichiometry of purified cytochrome *c* oxidase reconstituted in liposomes (COV) varies with the rate of electron flow and is depressed by the ΔpH component of the protonmotive force. A similar dependence of the H^+/e^- stoichiometry has also been observed for cytochrome *c* oxidase in the native mitochondrial membrane (Papa et al., 1991).

A large number of measurements of the H^+/e^- stoichiometry in COV have been carried out in different laboratories using the reductant pulse method. In this technique, in which COV equilibrated with air are pulsed with a molar excess of ferrocytochrome *c*, "optimal experimental conditions" are generally used which give H^+/e^- ratios approaching 1 (Wikström & Krab, 1979; Nicholls & Wriggelsworth, 1982; Proteau et al., 1983; Papa et al., 1987). It has, however, been found that with the pulse technique the H^+/e^- ratio varies with the pH, ionic composition of the medium, and modalities of activation of electron flow (Proteau et al., 1983; Papa et al., 1987). The reasons for these observations as well as their relation to the results of the present study remain to be clarified. It is possible that pulsing aerobic COV with an excess of ferrocytochrome *c* could generate a particular kinetic condition or state of the oxidase as well as give rise to some scalar acidification unrelated to proton pumping [see Papa et al. (1987)]. In any event the prevailing conditions used in the pulse method are such that this technique does not provide information on the aspects dealt with systematically in the present study, in which the H^+/e^- stoichiometry was analyzed with the rate method.

It is thought that, in order to be coupled to proton pumping in the oxidase, e^- flow has to follow the sequence cyt *c* → Cu_A → heme a → heme a_3-Cu_B (Babcock & Wikström, 1992). The binuclear center is the site where the oxygen reduction chemistry takes place and this process is generally considered to be intimately coupled to H^+ pumping. Mutational analysis (Hosler et al., 1993, 1994; Thomas et al., 1993) and X-ray crystallographic structure determination (Iwata et al., 1995; Tsukihara et al., 1995), have shown that heme a and the heme a_3-Cu_B binuclear center are bound in subunit I to histidine residues of transmembrane helices II, X, VI, and VII, in regions extending toward the N surface where, through loop XI−XII, they come in contact with the C-terminal domain of subunit II holding the two Cu_A (Iwata et al., 1995). Residues have been located in subunit II and the loop XI−XII of subunit I which might be involved in electron transfer from Cu_A to heme a (Iwata et al., 1995; Tsukihara et al., 1995). Several residues of the loop XI−

XII are in contact with the propionate groups of heme a. It can, however, be noted that heme a_3 is at about the same distance from the lower Cu_A atom as heme a (the distances between this Cu_A and the Fe of heme a and a_3 are 19.5 and 22.1 Å, respectively), and propionate groups of heme a_3 are also close to residues in the loop XI−XII (Iwata et al., 1995) as well as to other residues in subunit I on a possible way to Cu_A (Tsukihara et al., 1995).

Although proton pumping would be primarily coupled to oxygen reduction at the heme a_3-Cu_B center, a role in this process of heme a cannot be ruled out [cf. Babcock and Wikstrom (1992)].

Heme a exhibits Bohr effects (redox-linked p*K* shifts) (Wyman, 1968, Dutton & Wilson, 1974; Papa, 1976; Papa et al., 1979, 1994; Capitanio et al., 1990; Mitchell & Rich, 1994), and these could represent a cooperative device by which heme a participates in the pump by coupling electron delivery to the binuclear center with proton translocation from the N phase to this center.

Combining our results with the structural information available, we propose that e^- transfer from Cu_A to the heme a_3-Cu_B center can take place along two pathways. The first, via heme a, will be associated through short- and long-range cooperative linkage to uptake of H^+ from the N phase and its transfer to the binuclear center. These Bohr effects of heme a could thus allow the coupling of proton translocation to the chemistry of oxygen reduction and proton pumping to occur at the maximal efficiency of 1 H^+/e^-.

The second pathway will consist of direct electron transfer from Cu_A to the binuclear center, bypassing heme a and thus resulting in decoupling of the proton pump with an H^+/e^- ratio of 0. The occurrence of these two electron transfer pathways is supported by the observation that the reduction level of Cu_A is lower, in all the conditions tested, than that of heme a and is enhanced less than that of heme a by raising the rate of electron delivery to the oxidase (see Table 1).

The actual H^+/e^- in the oxidase will be determined by the relative contribution of the two electron transfer pathways and can vary from a maximum of 1 to 0 (Papa et al., 1994).

As shown by our results, the relative contributions of the two pathways can be dictated by kinetic and thermodynamic factors. Under level flow conditions, the H^+/e^- ratio should only be influenced by kinetic factors. At υ_i^{e-} below 4, the H^+/e^- ratio approaches 0. This might reflect the fact, that in order to perform proton pumping, the oxidase has to experience a full turnover, at least, with the passage of 4 electrons/mol of aa_3 (Babcock & Wikström, 1992). With enhancement of the rate of e^- flow, the H^+/e^- increases and, at intermediate rates, (υ_i^{e-} of around 10), tends to the maximal obtainable value of 1. The kinetic situation could be adjusted here to have the electrons flowing almost exclusively through the coupled pathway via heme a. As the electron pressure and the υ_i^{e-} of the oxidase are further increased, the uncoupled e^- transfer pathway, directly from Cu_A to the binuclear center, can start to become more important with a marked decrease in the H^+/e^- stoichiometry.

An additional way in which kinetic factors can influence the efficiency of the proton pump is represented by the fact that its operation requires the system to alternate between an H^+ input (H^+ being taken up by critical protolytic residues from the N phase) and an H^+ output state (H^+ being exchanged with the P phase; Malmström, 1985). When the rate of electron flow in the oxidase increases above a critical level, the speed at which the input−output states alternate can become inadequate to cope with that of e^- flow, with loss of the asymmetry of protonation−deprotonation and decoupling of the pump.

The other factor controlling the contribution of the two e^- transfer pathways is given by the transmembrane Δp. Our results show that transmembrane ΔpH markedly depresses the H^+/e^- stoichiometry. ΔpH can exert an inhibitory back-pressure on the proton-coupled e^- transfer pathway mediated by heme a without, obviously, affecting the uncoupled e^- transfer pathway, thus enhancing the contribution of the latter with consequent decrease of the H^+/e^- stoichiometry.

It should be noted that the loss of energy conversion in the proton pump of the oxidase effected by high respiratory rates and ΔpH is alleviated in mitochondria by two processes: (i) direct Δp generation arising from the membrane anisotropy of reduction of O_2 to H_2O in the oxidase and (ii) proton-coupled uptake of phosphate and respiratory substrates and proton influx for ATP synthesis in phosphorylating mitochondria which, similarly to what is shown here for CCCP in COV, contribute to prevent establishment of a large transmembrane ΔpH so that proton pumping at the steady state can be, as least in part, preserved.

At the steady state, alkalinization of the N phase can result in proton slip due to loss of protonation asymmetry of the critical protolytic group(s) in the pump in the input state. This protonation step might have a limited kinetic capacity (Hallen et al., 1992, 1994). An analogous situation seems to be met in the light-driven pump of bacteriorhodopsin, where the input proton pathway is the principal electrical barrier to the H^+ pump (Henderson et al., 1990). There are observations indicating that also this pump might "slip" at high Δp (Westerhoff & Dancshazy, 1984).

The detailed structure determination of cytochrome *c* oxidase provided by X-ray crystallographic analysis offers the rational basis for site-directed mutational analysis to verify our proposals, in particular the existence of two pathways for electron transfer from Cu_A to the binuclear center and the proposed role of heme a-linked proton transfer from the N phase to protonable groups in the heme a_3-Cu_B center.

Intrinsic decoupling (slip) in the proton pump of cytochrome *c* oxidase might be of physiological relevance. Recently, the rate dependence of the ATP/O ratio in yeast mitochondria has been investigated (Fitton et al., 1994). A decrease of the ATP/O ratio was observed when the electron flux was increased, which was ascribed essentially to a decrease in the H^+/e^- stoichiometry in cytochrome *c* oxidase.

It can be noted that the range of electron transfer in which the H^+/e^- stoichiometry varies in our experiments on COV corresponds to physiological respiratory rates of mitochondria in tissues (Fitzgerald, 1976), as well as to the rates of electron flow elicited by respiratory substrates in isolated mitochondria (Papa et al., 1991), both amounting to around 10% of the maximal turnover of cytochrome *c* oxidase. Under normal physiological conditions the oxidase utilizes, apparently, only a small fraction of its catalytic activity (Letellier et al., 1994).

Decoupling of the oxidase at high electron pressure and high Δp and phosphate potential could contribute to prevent excessive electronegativity of redox carriers in complex I and III, which, above a threshold level, can lead to production

of deleterious oxygen radicals. Enhanced oxidase activity will prevent accumulation of toxic $O_2^{\bullet -}$ and other free radicals deriving from it, also maintaining the cellular oxygen tension low (Skulachev, 1994) and possibly directly contributing to oxidation and/or dismutation of $O_2^{\bullet -}$ (Markossian et al., 1978).

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