Effect of Membrane Potential and pH Gradient on Electron Transfer in Cytochrome Oxidase†

Patricia McGovern Moroney,‡ Timothy A. Scholes, and Peter C. Hinkle*

ABSTRACT: Steady-state spectra of cytochrome oxidase in phospholipid vesicles were obtained by using hexaammineruthenium(II) and ascorbate as reductants. Cytochrome *a* was up to 80% reduced in the steady state in coupled vesicles. Upon addition of nigericin or acetate, which decrease ΔpH, resulting in an increase in $\Delta\psi$, cytochrome *a* became more oxidized in the steady state with no change in the rate of respiration. On the other hand, uncouplers or valinomycin plus nigericin, which lower both $\Delta\psi$ and ΔpH, stimulated respiration 2–8-fold and also lowered the steady-state level of reduction of cytochrome *a*. These experiments indicate that electron transfer between cytochromes *a* and a_3 is sensitive primarily to the pH gradient. Studies with the reconstituted and the soluble enzyme at various pH values indicated that the pH on the matrix side of the membrane, rather than ΔpH, controlled the steady-state level of reduced cytochrome *a*. Hexaammineruthenium(II) substituted for cytochrome *c* in measurements of proton pumping by cytochrome oxidase. Dicyclohexylcarbodiimide, which eliminated proton pumping by cytochrome oxidase, decreased the effect of ionophores on the steady-state level of reduced cytochrome *a*.

Although there is still controversy regarding the existence of proton transport in addition to transmembrane electron transfer in cytochrome oxidase (Mitchell & Moyle, 1983), the evidence for proton transport is substantial (Wikström & Krab, 1979), particularly in reconstituted cytochrome oxidase vesicles (Wikström & Saari, 1977; Sigel & Carafoli, 1978; Coin & Hinkle, 1979; Casey & Azzi, 1983). It appears that one proton is transported per electron transferred from cytochrome *c*, although it has been reported that two protons are transported per electron (Reynafarje et al., 1982).

Studies on the mechanism of proton transport by cytochrome oxidase have focused on cytochrome *a* and subunit III of the enzyme. The midpoint potential of cytochrome *a* is influenced by the pH inside mitochondria (Artzatbanov et al., 1978), and in its reduced form, cytochrome *a* shows a small energy-dependent spectral shift (Wikström, 1972). Subunit III of cytochrome oxidase has been implicated in proton transport more directly. When subunit III is covalently labeled with *N,N'*-dicyclohexylcarbodiimide (DCCD)[1] (Casey et al., 1980) or is removed from the enzyme (Saraste et al., 1981), the reconstituted oxidase no longer translocates protons. In cytochrome oxidase without subunit III, the midpoint potential of cytochrome *a* is no longer sensitive to pH (Penttilä, 1983).

We have investigated the mechanism of energy coupling in cytochrome oxidase by measuring the effect of the electrochemical proton gradient on the steady-state spectra of tightly coupled cytochrome oxidase vesicles (with respiratory control ratios greater than 8). Hexaammineruthenium(II) was used as the reductant in these experiments. Scott & Gray (1980) used hexaammineruthenium(II) as a substitute for cytochrome *c* in the reduction of cytochrome oxidase. As with cytochrome *c*, the steady-state kinetics of the reaction of cytochrome oxidase with hexaammineruthenium(II) gave nonlinear Eadie–Hofstee plots, and cytochrome *c* was competitive with hexaammineruthenium(II) for this reaction (Hochman et al., 1981). Since hexaammineruthenium(II) has little visible absorbance, it is more suitable as a reductant than cytochrome *c* for spectral measurements of cytochrome oxidase. The steady-state spectra reported here indicate that electron transfer between cytochrome *a* and cytochrome a_3 was inhibited by the electrochemical proton gradient. This inhibition was caused primarily by the pH on the matrix side of the enzyme.

Experimental Procedures

Materials. DCCD was a product of Schwarz/Mann. Hexaammineruthenium(III) chloride was purchased from Alfa and recrystallized (Pladziewicz et al., 1973). Cytochrome *c* type VI was obtained from Sigma; the reduced form was prepared at a concentration of about 10 mM by treatment with a 4-fold excess of potassium ascorbate followed by passage over a Sephadex G-10 column. Soybean phospholipids from Sigma were acetone washed as described by Kagawa & Racker (1971) but without antioxidant. Cholic acid from Sigma was recrystallized (Schneider et al., 1971).

Preparations. Cytochrome oxidase was prepared by the method of Yonetani (1966) except that additional ammonium sulfate fractionations were carried out in 0.1 M sodium phosphate–1% Tween 80, pH 7.4. Cytochrome oxidase vesicles were made by cholate dialysis (Hinkle et al., 1972): acetone-washed soybean phospholipids at 30 mg/mL were sonicated to clarity in a solution of 1.5% cholate–50 mM potassium phosphate, pH 7.4. Cytochrome oxidase was added to give 10 μM heme *a*, and the solution was dialyzed against 50 mM potassium phosphate, pH 7.4, with a change after 4 h to either 50 mM potassium phosphate, pH 7.4, or 50 mM HEPES, 25 mM KCl, and 1 mM EDTA, pH 7.4, or 50 mM K_2SO_4 and 0.2 mM MOPS, pH 7.4. DCCD-treated cytochrome oxidase vesicles were prepared as follows: 37.5 μL of 100 mM DCCD (in methanol) was added to 1.5 mL of cytochrome oxidase vesicles dialyzed against 50 mM HEPES,

† From the Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853. *Received January 11, 1984; revised manuscript received May 8, 1984.* This work was supported by Research Grant HL-14483 from the National Institutes of Health.

‡ Supported by a National Science Foundation Predoctoral Fellowship.

[1] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; 1799, bis(hexafluoroacetonyl)acetone; SF6847, (3,5-di-*tert*-butyl-4-hydroxybenzylidene)malononitrile; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; MES, 2-(*N*-morpholino)-ethanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

0006-2960/84/0423-4991$01.50/0

25 mM KCl, and 1 mM EDTA, pH 7.4. The solution was incubated for 3 h at 20 °C and then passed over a Sephadex G-50 fine column or "centri-column" (Penefsky, 1979) to remove unbound DCCD. Control vesicles received the same treatment except that 37.5 μL of methanol was added instead of the DCCD solution.

Optical Measurements. Spectra were recorded in a Cary 219 spectrophotometer. The level of heme *a* reduction was measured at 605–630 nm in an Aminco DW-2 or Aminco Britton Chance dual-wavelength spectrophotometer. Flow–flash experiments were modeled after Greenwood & Gibson (1967). A 1.2-cm path-length Plexiglass observation cell was fitted to the Aminco-Morrow stopped-flow apparatus. A Corning glass filter (3-66) was placed between the 250-J xenon flash lamp and the observation cell, and two blue filters (Oriel 5182 and 5183) were placed between the observation cell and the photomultiplier tube (Hammamatsu R928) to screen out light from the flash. In this configuration, the carbon monoxide–cytochrome oxidase complex was photolyzed with red light, and the reaction was observed at 445 nm. To study the reaction of reduced cytochrome oxidase with oxygen, a solution of the CO complex of reduced cytochrome oxidase in detergent solution was mixed with oxygen-containing buffer in the modified stopped-flow apparatus. The xenon flash was fired 8 ms after mixing to displace CO from the enzyme, and oxidation of the hemes was followed by 445 nm. The fast and slow phases contribute approximately 60% and 40%, respectively, to the total absorbance change at 445 nm.

Proton Transport. Proton transport by cytochrome oxidase vesicles was measured with a combination pH electrode either by reduced cytochrome *c* pulses or by oxygen pulses in a 2-mL thermostated glass cell stirred magnetically. An Analog Devices 311J electrometer and chart recorder were arranged to give 0.1 pH unit full-scale. For oxygen pulses (5 or 10 μL of air-equilibrated medium), the medium was made anaerobic by bubbling with N_2 prior to the experiment, and N_2 was continuously blown over the surface of the medium during the experiment.

Oxygen Measurements. Respiration rates and respiratory control ratios for cytochrome oxidase vesicles were determined polarographically at 25 °C with a Yellow Springs Instrument Co. Model LN5320 oxygen electrode in a 1.5-mL glass chamber. The assay mixture contained 15 mM potassium ascorbate, 63 μM cytochrome *c*, and 70 nM heme *a* (cytochrome oxidase vesicles) in 50 mM potassium phosphate, pH 7.4. Valinomycin and SF6847 were added at concentrations of 0.28 and 1.3 μM, respectively, to uncouple respiration.

The rate of turnover for cytochrome oxidase in vesicles with hexaammineruthenium(II) as reductant in the presence of uncouplers was 50 mol of electrons s^{-1} (mol of oxidase)$^{-1}$ under the following conditions: 50 mM HEPES, 25 mM KCl, pH 7.4, 3 mM potassium ascorbate, 310 μM hexaammineruthenium(III) chloride, 0.25 μM heme *a* (cytochrome oxidase vesicles), 0.28 μM valinomycin, and 1.3 μM SF6847. The rate of turnover for cytochrome oxidase in detergent solution was 8 mol of electrons s^{-1} (mol of oxidase)$^{-1}$ under the following conditions: 50 mM HEPES, 1% Tween 80, pH 7.4, 63 μM hexaammineruthenium(III) chloride, and 0.1 μM heme *a*. The rates were corrected for oxygen uptake in the presence of 1 mM KCN due to hexaammineruthenium(II) autoxidation.

Other Analytical Methods. Heme *a* concentration was determined by using $\Delta\epsilon_{605}$(reduced − oxidized) = 12 mM^{-1} cm^{-1} (Kuboyama et al., 1972). Cytochrome *c* concentration was determined by using $\Delta\epsilon_{550}$(reduced − oxidized) = 21 mM^{-1} cm^{-1} (Massey, 1959).

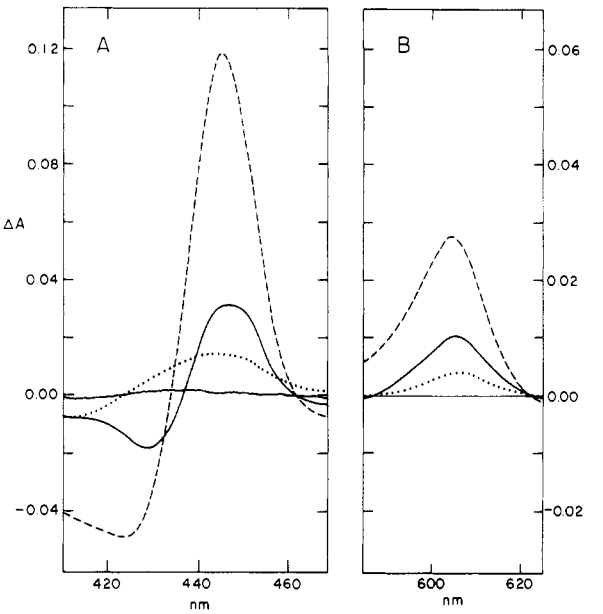

FIGURE 1: Steady-state difference spectra of cytochrome oxidase vesicles in the absence and presence of uncouplers. *Solid curve*, (A) 1.5 μM heme *a* (cytochrome oxidase vesicles), 250 μM hexaammineruthenium(III) chloride, 50 mM HEPES, and 25 mM KCl, pH 7.4; (B) 2.3 μM heme *a* (cytochrome oxidase vesicles), 250 μM hexaammineruthenium(III) chloride, and 50 mM potassium phosphate, pH 7.4. Potassium ascorbate (2.5 mM) was added, and the steady-state difference spectrum vs. oxidized cytochrome oxidase vesicles was recorded. *Dotted curve*, same as above but (A) 1799 was added at concentration of 7 μM or (B) valinomycin and nigericin were added at concentrations of 0.18 and 0.28 μM, respectively, before addition of potassium ascorbate for the uncoupled steady-state spectra. *Dashed curve*, solid sodium dithionite was added to generate the reduced minus oxidized difference spectrum. Spectra were measured in a Cary 219 spectrophotometer. The spectra in (B) were corrected for small base-line changes.

Results

Steady-state spectra have previously been used to identify coupling sites in cytochrome oxidase. Chance & Williams (1956) demonstrated upon the transition from state 3 (ADP present) to state 4 (ADP consumed) in mitochondria respiring in the steady state, cytochromes $c + c_1$ became more reduced while cytochromes $a + a_3$ became more oxidized. This crossover point was interpreted to be a site of coupling between respiration and ADP phosphorylation. Wrigglesworth & Nicholls (1978) measured the steady-state spectra of respiring cytochrome oxidase vesicles using ascorbate, TMPD, and cytochrome *c* as reductants. They found that addition of uncouplers to the vesicles resulted in oxidation of cytochrome *c* and reduction of cytochrome *a*.

Examples of steady-state minus oxidized difference spectra of cytochrome oxidase in vesicles obtained by using ascorbate and hexaammineruthenium(II) as reductants are shown in Figure 1. For comparison, a reduced minus oxidized spectrum (dashed curve) generated by using sodium dithionite is also shown (Figure 1). For the steady-state spectrum (solid curve), the peak at 446 nm, trough at 427 nm, and isosbestic point at 436.5 nm correspond to those of the kinetic difference spectrum for the fast phase of reduction of detergent-solubilized cytochrome oxidase. Scott & Gray (1980) measured values of 445, 425, and 435 nm and Reichardt & Gibson (1982) found 448, 428, and 437.5 nm for the peak, trough, and isosbestic points, respectively, of the fast phase of reduction. This phase has been attributed to cytochrome *a* reduction (Scott & Gray, 1980). In addition, the ratio of A_{445}/A_{605} we obtained in steady-state spectra of cytochrome

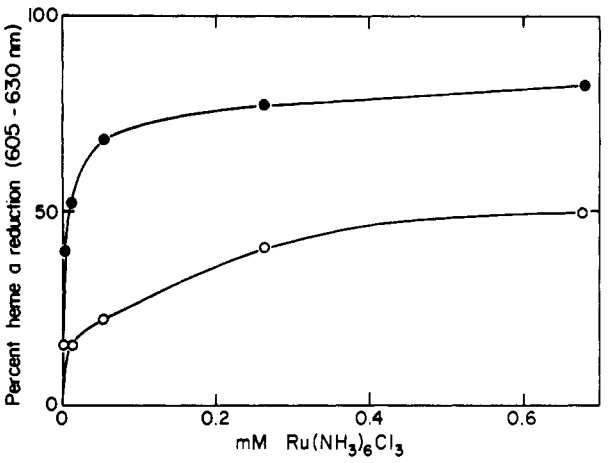

FIGURE 2: Dependence of the steady-state level of reduced cytochrome *a* on the hexaammineruthenium(III) chloride concentration. Conditions: 0.49 μM heme *a* (cytochrome oxidase vesicles), 10 mM potassium ascorbate, and hexaammineruthenium(III) chloride at the indicated concentrations in 1.2 mL of 50 mM HEPES, 25 mM KCl, and 1 mM EDTA, pH 7.4. Percent heme *a* reduction was the percentage of the maximum reduced minus oxidized $A_{605-630}$ measured in an Aminco DW-2 spectrophotometer. (●) No addition; (○) in the presence of 6.5 μM 1799.

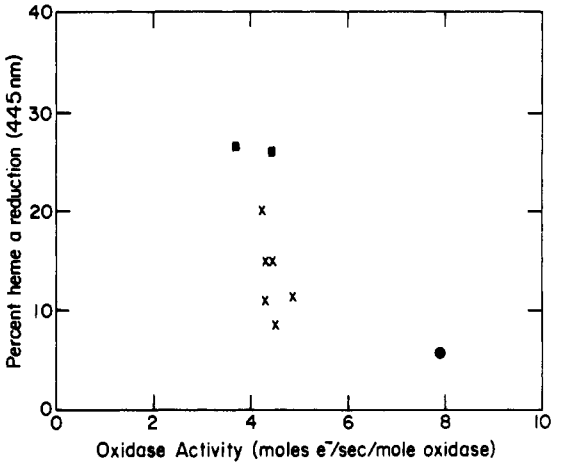

FIGURE 3: Effect of potassium acetate and 1799 on the steady-state level of reduced heme *a* (at 445 nm) and on the respiration rate. Conditions: 1.5 μM heme *a* (cytochrome oxidase vesicles), 2.4 mM potassium ascorbate, and 250 μM hexaammineruthenium(III) chloride in 1.0 mL of 50 mM HEPES, 25 mM KCl, and 1 mM EDTA, pH 7.4. Percent heme *a* reduction was the percentage of the maximum reduced minus oxidized A_{445} measured in a Cary 219 spectrophotometer. Respiration rates were measured polarographically in a 1.5-mL cell under the same conditions as the absorbance measurements except that the rates were corrected for oxygen uptake in the presence of 1 mM KCN due to hexaammineruthenium(II) autoxidation. The correction was 3 mol of electrons s^{-1} (mol of oxidase)$^{-1}$. (■) No additions; (×) 2, 5, 10, 30, and 60 mM potassium acetate; (●) 8 μM 1799.

oxidase vesicles was 2.8, close to the value of 3.8 found for cytochrome *a* by Gibson et al. (1965) in reductive experiments with the solubilized enzyme. For comparison, they found that the A_{445}/A_{605} ratio was 16 for cytochrome a_3. Thus, these spectra indicate that cytochrome *a* is partially reduced and cytochrome a_3 is oxidized in the steady state. Figure 1 shows that when an uncoupler is added to collapse the electrochemical proton gradient, the steady-state absorbance at 446 nm decreases, indicating oxidation of cytochrome *a* (Figure 1, dotted curve). These results imply that the electrochemical proton gradient inhibits electron transfer between cytochromes *a* and a_3.

This result is different from the observations of Wrigglesworth & Nicholls (1978), who recorded the steady-state spectra of cytochrome oxidase in vesicles prepared by sonication using ascorbate, TMPD, and cytochrome *c* as reductants. In their studies, cytochrome *a* became more reduced and cytochrome *c* more oxidized upon addition of uncouplers, indicating a crossover point between cytochromes *c* and *a*. We repeated these experiments under their conditions except using cytochrome oxidase vesicles prepared by cholate dialysis. Unlike Wrigglesworth and Nicholls, we found that both cytochrome *c* and cytochrome *a* became more oxidized upon addition of uncoupler. Differences between their experiment and ours were the method of vesicle preparation and the method of oxidase preparation. Sonication vesicles have a random orientation of cytochrome oxidase across the membrane (Wrigglesworth, 1978) and showed a respiratory control ratio of 2.8 (Wrigglesworth & Nicholls, 1978); in cholate dialysis vesicles, cytochrome oxidase is predominantly in the mitochondrial configuration (Carroll & Racker, 1977; Nicholls et al., 1980), and the respiratory control ratio we obtained was 8.3.

Measurements of the steady-state level of reduction of cytochrome *a* at different concentrations of hexaammineruthenium in the absence and presence of uncoupler are shown in Figure 2. Approximately 50 times higher concentrations of hexaammineruthenium are required to reach the same level of reduction in the presence of 1799 compared to the coupled steady state.

The effects of ionophores and potassium acetate on the steady-state spectra were examined in order to determine the relative effects of $\Delta\psi$ and ΔpH on the steady-state level of reduced cytochrome *a*. Since acetic acid is a membrane-permeant acid, addition of potassium acetate to respiring cytochrome oxidase vesicles should decrease the pH gradient. Potassium acetate was added to cytochrome oxidase vesicles respiring with ascorbate and hexaammineruthenium(II), and the steady-state level of reduction at 445 nm was recorded. Cytochrome oxidase activity was measured polarographically under the same conditions. Figure 3 shows an analysis of measurements of the level of heme *a* reduction and respiration in the presence of potassium acetate at concentrations up to 60 mM. As the concentration of potassium acetate was increased, the level of heme *a* reduction decreased with little or no change in the rate of respiration. In the medium of 50 mM HEPES, 25 mM KCl, and 1 mM EDTA, pH 7.4, even 2, 5, and 10 mM potassium acetate significantly reduced the steady-state level of heme *a* reduction, indicating that the potassium acetate effect is not due to increasing ionic strength. For comparison, the values obtained in the presence of the uncoupler 1799 are plotted. 1799 decreased the steady-state absorbance at 445 nm, but an increase in the rate of respiration was also observed. The stimulation in oxidase activity was only about 2-fold due to the fact that these conditions were not optimal for determining respiratory control ratios.

Further experiments were conducted by using the absorbance at 605 nm minus that at 630 nm to monitor the steady-state redox level of cytochrome *a*. Figure 4 shows the steady-state level of cytochrome *a* reduction and respiration rates in the presence of the ionophores valinomycin, nigericin, and 1799. The membrane potential created by cytochrome oxidase in the reconstituted vesicles is transient and decreased in a few seconds as ΔpH increased, as measured with a cyanine dye (Tarba, 1978, 1983). Addition of nigericin or acetate decreased ΔpH and restored the membrane potential, whereas valinomycin caused a small decrease in $\Delta\psi$ and an increase in ΔpH. Valinomycin at concentrations of 15 nM–0.8 μM had no effect on the rate of respiration or the steady-state level

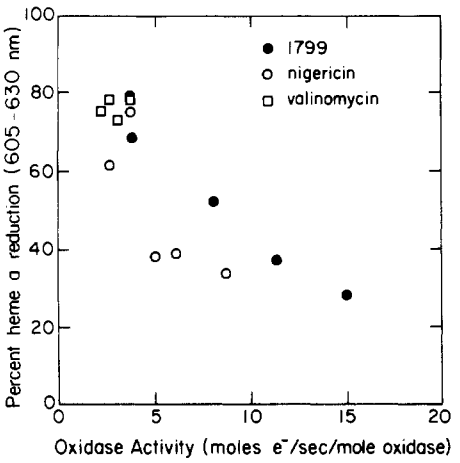

FIGURE 4: Effect of ionophores on the steady-state level of reduced heme *a* (at 605 – 630 nm) and on the respiration rate. Conditions: 0.44 μM heme *a* (cytochrome oxidase vesicles), 2 mM potassium ascorbate, and 210 μM hexaammineruthenium(III) chloride in 1.2 mL of 50 mM HEPES, 25 mM KCl, and 1 mM EDTA, pH 7.4. Percent heme *a* reduction was the percentage of the maximum reduced minus oxidized $A_{605-630}$ measured in an American Instrument Co. Britton Chance dual-wavelength spectrophotometer. Respiration rates were measured polarographically under the same conditions as the absorbance measurements except that the rates were corrected for oxygen uptake in the presence of 1 mM KCN due to hexaammineruthenium(II) autoxidation. The correction was equivalent to 17 mol of electrons s^{-1} (mol of oxidase)$^{-1}$. (●) 0, 65 nM, 0.7 μM, 2.9 μM, and 9.4 μM 1799; (□) 0, 15 nM, 0.17 μM, and 0.9 μM valinomycin; (○) 0, 2 nM, 12 nM, 46 nM, and 0.6 μM nigericin. The percent heme *a* reduction decreased with increasing concentrations of 1799 or nigericin.

of cytochrome *a* reduction. Nigericin decreased the level of reduced cytochrome *a* in the steady state, as did the proton ionophore 1799. However, nigericin did not stimulate respiration except at high concentrations where it is probably also acting by an electrogenic mechanism (Pressman, 1976) and thus decreasing both $\Delta\psi$ and ΔpH.

Figures 3 and 4 indicate that nigericin and potassium acetate decreased the steady-state level of reduced cytochrome *a* but had little or no effect on the respiration rate. This suggests that the rate-limiting step in respiration changed when the pH gradient decreased. Moreover, the observation that the rate of respiration remained the same implies that the electrochemical proton gradient ($\Delta\psi$ + ΔpH) remained approximately constant and that the membrane potential increased as the pH gradient decreased. The level of reduced cytochrome *a* decreased under these conditions. From this, it appears that electron transfer from cytochrome *a* to cytochrome a_3 is sensitive primarily to the pH gradient rather than the membrane potential.

DCCD binds to subunit III of cytochrome oxidase and inhibits proton pumping (Casey et al., 1980; Prochaska et al., 1981). In our hands, DCCD treatment (2.5 mM DCCD, 3 h at 20 °C followed by passage through Sephadex G-50 to remove unreacted DCCD) inhibited the proton ejection by 84% following a pulse of reduced cytochrome *c* to cytochrome oxidase vesicles whereas the rate of respiration in the presence of uncouplers was inhibited 50%, and the respiratory control ratio declined only slightly from 8.4 to 6.9.

The proton pumping activity of cytochrome oxidase incorporated in liposomes was also tested with hexaammineruthenium(II) as substrate. Since hexaammineruthenium(II) reacts with O_2, an O_2 pulse procedure was used. This kept the O_2 concentration low and minimized the nonenzymatic reaction. Typical oxygen pulses with normal and DCCD-treated cytochrome oxidase vesicles are shown in Figure 5.

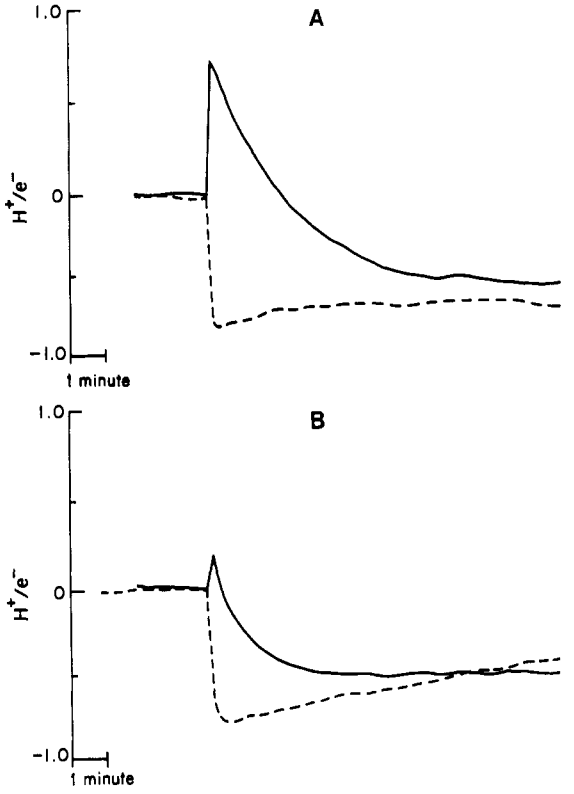

FIGURE 5: Proton transport with hexaammineruthenium as substrate by (A) control and (B) DCCD-treated cytochrome oxidase vesicles. Measurements were made as described under Experimental Procedures in a medium of 50 mM K_2SO_4, 0.2 mM MOPS, 1 μM heme *a* (cytochrome oxidase vesicles), 0.25 μg/mL valinomycin, 3 mM potassium ascorbate, and 50 μM hexaammineruthenium(III). The dashed lines are in the presence of 1 μM SF6847.

The reaction in the presence of the uncoupler SF6847 is caused by the scalar uptake of protons to form H_2O (H^+/e^- = −1 is expected) followed by the slow reduction of hexaammineruthenium(III) by ascorbate (H^+/e^- = +0.5 is expected). The scalar proton uptake was 85% of the expected value for reasons that are not clear but might include a small amount of acid formation from binding of hexaammineruthenium to the negatively charged phospholipids present. In other experiments, we formed vesicles with cytochrome oxidase plus the zwitterionic phospholipids phosphatidylcholine and phosphatidylethanolamine (1:4) as described (Carroll & Racker, 1977) and found an initial acid formation of 0.5 H^+/e^- in the presence of 3 mM dithiothreitol and 50 μM hexaammineruthenium(II). The acid formation following an oxygen pulse in Figure 5 is clear evidence for proton efflux catalyzed by cytochrome oxidase with hexaammineruthenium(II) as substrate. DCCD treatment reduced the acid phase by 73%, similar to results with cytochrome *c* as substrate.

The experiments of Figures 3 and 4 show that reagents which decrease ΔpH also decrease the level of reduced cytochrome *a* in the steady state, indicating an increase in the rate of electron transfer from cytochrome *a* to cytochrome a_3 when ΔpH is small. To determine whether this effect requires a pH gradient (ΔpH) or is simply an effect of the internal pH alone, we measured the steady-state level of cytochrome *a* reduction at various pH values in the presence of the uncoupler SF6847 so that there would be no ΔpH (Figure 6). The steady state was constant from pH 6 to 7.5, but above pH 7.5, there was a significant increase in the reduction of cytochrome *a*. DCCD caused cytochrome *a* to be largely reduced in the steady state at all pH values. Studies with the soluble enzyme in Tween 80 (not shown) also showed an increase in the steady-state level

Table I: Effect of DCCD Treatment on Cytochrome Oxidase Vesicles

	% heme *a* reduction			respiratory control ratio	respiration rate [mol of electrons s^{-1} (mol of oxidase)$^{-1}$]
	alone	+1799	+nigericin		
control	75	30	38	8.4	164
DCCD treated	84	64	67	6.9	86

[a] Control and DCCD-treated vesicles were prepared as described under Experimental Procedures. Percent heme *a* reduction was the steady-state percentage of the maximum reduced minus oxidized $A_{605-630}$ measured in an Aminco DW-2 spectrophotometer. The total $\Delta A_{605-630}$ for reduced minus oxidized was 0.006 for both control and DCCD-treated vesicles. Conditions: 0.49 μM heme *a* (cytochrome oxidase vesicles), 2 mM potassium ascorbate, 208 μM hexaammineruthenium chloride, 50 mM HEPES, 25 mM KCl, and 1 mM EDTA, pH 7.4. The uncoupler 1799 was added to give 6.5 μM and nigericin was added to give 12 nM. Respiratory control and respiration rates were measured with cytochrome *c* as substrate as described under Experimental Procedures.

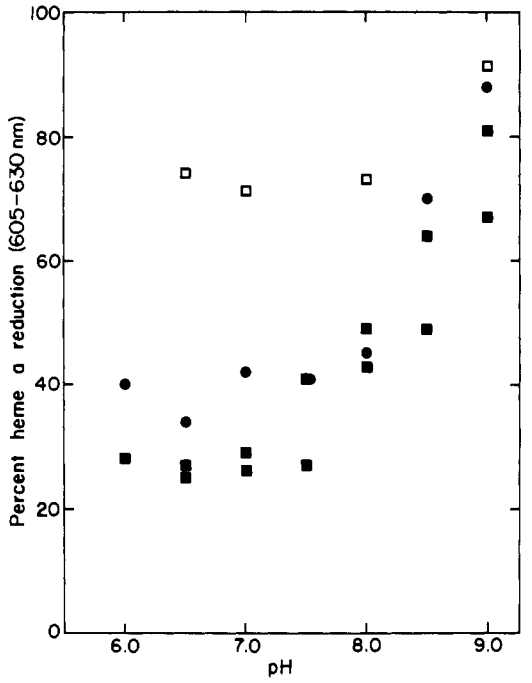

FIGURE 6: Steady-state reduction level of cytochrome *a* as a function of pH. Cytochrome oxidase vesicles treated with DCCD (□) and untreated (●) and control (■) vesicles were suspended at 1.0 μM heme *a* in 1.0 mL of 100 mM KCl, 50 mM buffer, 1 μg/mL valinomycin, and 1 μM SF6847. The buffers used were MES at pH 6.0 and 6.5, MOPS at pH 7.0, HEPES at pH 7.5, TAPS at pH 8.0 and 8.5, and CHES at pH 9.0. Sodium ascorbate (10 mM) was added to give the oxidized base line and hexaammineruthenium (0.1 mM) to give the steady-state level shown. After 1–5 min, the system became anaerobic, and that level of reduction was taken to be 100%.

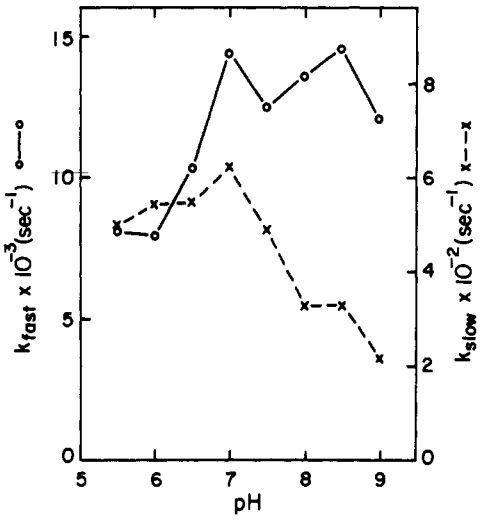

FIGURE 7: pH dependence of the fast and slow phases of the reaction of oxygen with reduced cytochrome oxidase. The reaction rates were determined by the flow–flash technique (Experimental Procedures). Concentrations after mixing were 1.2 μM heme *a*, 10 mM potassium ascorbate, 0.13 μM cytochrome *c*, 0.25% Tween 80, 130 μM O_2, 16 μM CO, and 50 mM buffer at the indicated pH. The buffers were the following: at pH 5.5, citrate; at pH 6.0 and 6.5, MES; at pH 7.0 and 7.5, MOPS; at pH 8.0 and 8.5, TAPS; at pH 9.0, CHES. The temperature was 25 °C. The xenon flash was fired 8 ms after the end of flow.

of cytochrome *a* on treatment with DCCD at pH 7.5. Table I shows the effect of DCCD on the steady-state level of reduced cytochrome *a* in cytochrome oxidase vesicles in the absence and presence of uncouplers. DCCD treatment caused a small increase in the level of reduction of cytochrome *a*. More striking, however, is the decrease in response to 1799 and nigericin in the DCCD-inhibited cytochrome oxidase vesicles. It appears that DCCD inhibited electron transfer between cytochrome *a* and cytochrome a_3.

The sensitivity to pH of electron transfer from cytochrome *a* to cytochrome a_3 was also studied by conducting flow–flash experiments with the soluble enzyme at pH values from 5.5 to 9 at 130 μM O_2. Figure 7 shows the rates of the fast and slow phases of heme *a* oxidation vs. pH. There was a 3-fold decrease in the rate of the slow phase when the pH was increased from 7 to 9. Although there is ambiguity in the assignment of the two phases in flow–flash experiments to a particular reaction (Reichardt & Gibson, 1982), Hill & Greenwood (1984) have recently presented evidence that 40% of the cytochrome *a* is oxidized along with cytochrome a_3 in the fast phase of the reaction with O_2 and the remainder in the slow phase. The slow phase of heme oxidation may correspond to the step which is inhibited by high internal pH in the steady-state experiments with cytochrome oxidase vesicles.

Discussion

Steady-state spectra of tightly coupled cytochrome oxidase vesicles obtained by using ascorbate and hexaammineruthenium(II) as reductants showed that electron transfer between cytochromes *a* and a_3 was influenced by the magnitude and nature of the electrochemical proton gradient. Nigericin or potassium acetate decreased the level of reduced cytochrome *a* in the steady state without increasing the rate of respiration (Figures 3 and 4), indicating that the major effect of the electrochemical proton gradient on electron transfer from cytochrome *a* is due to the pH gradient rather than the membrane potential. The pH inside appears to be the controlling factor since steady-state measurements with uncoupled vesicles (Figure 6) and soluble oxidase showed that the level of reduction of cytochrome *a* is pH dependent. DCCD, which inhibited proton translocation (Figure 5), also inhibited electron transfer between cytochrome *a* and cytochrome a_3 (Figure 6, Table I). The steady-state cytochrome *a* level measurements do not reflect properties of the resting enzyme or the transition of resting to pulsed enzyme (Antonini et al., 1977) because the same level was reached when oxygen was added to the reduced enzyme as when substrates were added to the oxidized enzyme. As reported by Antonini et

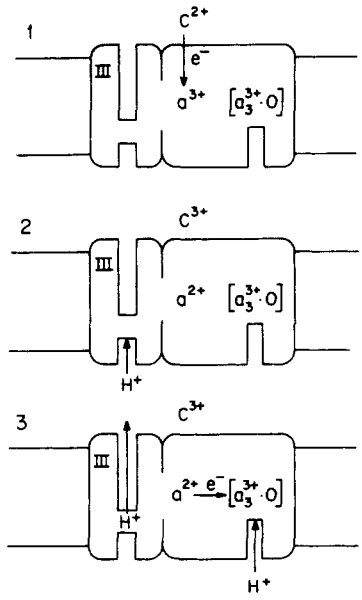

FIGURE 8: Proposed mechanism of proton translocation in cytochrome oxidase. Step 1, an electron is transferred from cytochrome *c* to cytochrome *a*. Step 2, a proton binds to subunit III. Step 3, an electron is transferred from cytochrome *a* to cytochrome a_3, a proton is ejected into the cytoplasmic phase, and the oxygen intermediate is protonated from the matrix phase.

al. (1977), at high reductant levels the transition to the pulsed form is rapid.

Our results can be interpreted in terms of a modified version of the scheme first proposed by Artzatbanov et al. (1978) shown in Figure 8. Step 1 of Figure 8 is electrogenic electron transfer from reduced cytochrome *c* on the outside of the membrane to cytochrome *a* buried in the membrane. Step 2 involves protonation of the enzyme (cytochrome *a* reduced) from the inside of the membrane. Step 3 shows subsequent electron transfer and proton translocation required so that the three steps will account for the stoichiometry per electron of two protons taken up from the matrix side of the membrane and one proton appearing on the cytoplasmic site; two charges per electron cross the membrane in this process [Wikström & Krab, 1979; but see also Reynafarje et al., (1982)]. Step 3 indicates that proton movement is responsible for transmembrane charge transfer, but this could also be due in part to electron transfer, depending on the location of cytochrome *a* and cytochrome a_3.

The binding of a proton to cytochrome oxidase when cytochrome *a* is reduced (step 2, Figure 8) was first indicated by the pH dependence of the midpoint potential of cytochrome *a* in equilibrium redox titrations conducted anaerobically (Wilson et al., 1972) or with the azide- or cyanide-inhibited enzyme (Wilson et al., 1976; Artzatbanov et al., 1978). Artzatbanov et al. (1978) showed that the midpoint potential of cytochrome *a* was sensitive to the pH inside the mitochondria. Thus, the observation that the level of reduced cytochrome *a* in the steady state is sensitive primarily to the pH inside (Figures 3, 4, and 6) can be explained in terms of step 2 of Figure 8, in which a proton must bind to cytochrome oxidase before cytochrome *a* can transfer its electron, although the steady-state effect occurs only at high pH whereas the effect on E_m occurs at least down to pH 6.5 (Penttilä, 1983). We found no evidence for transmembrane charge transfer at this step. Nigericin or potassium acetate increased $\Delta\psi$ at the expense of ΔpH, yet the inhibition of electron transfer from cytochrome *a* was relieved, indicating that ΔpH was primarily responsible for the inhibition. In addition, measurements of the oxidation of the hemes by the flow–flash technique of Greenwood & Gibson (1967) showed no difference in the rates of oxidation in the absence or presence of a valinomycin-induced membrane potential (data not shown). Since electron transfer from cytochrome *a* is primarily sensitive to the pH inside, this implies that the protonation in step 2 does not involve movement of the proton across the membrane or into a proton well, since these events should be sensitive to both $\Delta\psi$ and ΔpH. It is possible, however, that the movement of charge was too small to influence significantly the rates of electron transfer in these experiments. The observation that DCCD, which inhibited proton pumping, decreased the effect of uncouplers on the steady-state level of reduction of cytochrome *a* (Table I) indicates that the proton binding is a step in proton pumping rather than in water formation.

These experiments also suggest a mechanism for the inhibition of proton translocation caused by DCCD. Since DCCD inhibition of the enzyme mimics the effects of high pH inside on the steady-state level of reduced cytochrome *a* (Figure 6, Table I), DCCD may inhibit the binding of a proton to the matrix side of the enzyme (step 2, Figure 8). Although Penttilä & Wikström (1981) showed that DCCD has no effect on the pH dependence of the midpoint potential of cytochrome *a*, it is possible that DCCD affects the rate of proton binding in step 2 but not the pK_a of the group responsible. If DCCD changed the rate of protonation in step 2, this would be reflected in the steady-state level of reduction of cytochrome *a*, while the pK_a of the acid or base group, which would be reflected in the equilibrium redox titration, could remain the same. Alternatively, DCCD may inhibit proton ejection into the cytoplasmic phase (step 3) which could also slow electron transfer from cytochrome *a*.

The transmembrane electron transfer of step 1 (Figure 8) follows from the experiments of Hinkle & Mitchell (1970), who showed that the midpoint potential of cytochrome *a* changed by about half the membrane potential in carbon monoxide inhibited mitochondria in which cytochrome a_3 is reduced. They ruled out that the charge movement is due to proton movement at this step since the midpoint potential of cytochrome *a* was membrane potential dependent in carbon monoxide inhibited cytochrome oxidase even when the electrochemical potential of the proton across the membrane was zero due to the presence of an uncoupler (Hinkle & Mitchell, 1970). We have found that the midpoint potential of cytochrome *a* is also membrane potential dependent in cyanide-inhibited cytochrome oxidase vesicles, in which cytochrome a_3 is oxidized (data not shown). The actual location of heme *a* in the membrane is not shown by these experiments, however. The factor of half $\Delta\psi$ indicates an "electrical distance" which depends on the local dielectric and ionic structure.

From the evidence cited above, it appears that the sequence of catalysis in cytochrome oxidase contains steps which are primarily $\Delta\psi$ dependent (step 1) or ΔpH dependent (step 2). On the other hand, it would be expected that movement of a proton in a proton well (Mitchell, 1968) would be sensitive to both $\Delta\psi$ and ΔpH. A membrane potential, positive outside, would make the local pH more acid at the bottom of a well which leads to the outer aqueous phase and more basic at the bottom of a well which leads to the inner aqueous phase. The kinetics of proton movement in such proton wells may be complex, however, and have been studied directly only in TF_0 (Sone et al., 1981) where the system appeared to have a K_m for protons. The processes in step 3 may be too fast to become rate limiting even in the presence of an electrochemical proton gradient since we were unable to detect them in steady-state

experiments or flow-flash experiments using a potassium gradient and valinomycin-generated membrane potential. Chance et al. (1975) reported similar results in low-temperature experiments of the "triple trap" type in mitochondria. ATP had little influence on the rate of formation of the first intermediate but did appear to slow electron transfer from cytochrome *a* and cytochrome *c*. Wikström (1981) found that it is possible to reverse electron transfer at cytochrome a_3 by the addition of ATP to mitochondria at high oxidation potentials. A study of this phenomenon may provide information on the sequence of events in step 3.

The steady-state measurements reported here have shown that the membrane potential and pH gradient exert disparate effects on electron transfer in cytochrome oxidase. These results are compatible with the transmembrane charge transfer and protonation/deprotonation which are the elements of a proton pump.

Registry No. H^+, 12408-02-5; cytochrome oxidase, 9001-16-5.

References

Antonini, E., Rrunori, M., Colosimo, A., Greenwood, C., & Wilson, M. T. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3128–3132.

Artzatbanov, V. Yu., Konstantinov, A. A., & Skulachev, V. P. (1978) *FEBS Lett. 87*, 180–185.

Carroll, R. C., & Racker, E. (1977) *J. Biol. Chem. 252*, 6981–6990.

Casey, R. P., & Azzi, A. (1983) *FEBS Lett. 154*, 237–242.

Casey, R. P., Thelen, M., & Azzi, A. (1980) *J. Biol. Chem. 255*, 3994–4000.

Chance, B., & Williams, G. R. (1956) *Adv. Enzymol. Relat. Areas Mol. Biol. 17*, 65–134.

Chance, B., Harmon, J., & Wikström, M. (1975) in *Electron Transfer Chains and Oxidative Phosphorylation* (Quagliariello, E., et al., Eds.) pp 81–95, North-Holland Publishing Co., Amsterdam.

Coin, J. T., & Hinkle, P. C. (1979) in *Membrane Bioenergetics* (Lee, C. P., et al., Eds.) pp 405–412, Addison-Wesley, Reading, MA.

Gibson, Q. H., Greenwood, C., Wharton, D. C., & Palmer, G. (1965) *J. Biol. Chem. 240*, 888–894.

Greenwood, C., & Gibson, Q. H. (1967) *J. Biol. Chem. 242*, 1782–1787.

Hill, B. C., & Greenwood, C. (1984) *FEBS Lett. 166*, 362–366.

Hinkle, P., & Mitchell, P. (1970) *J. Bioenerg. 1*, 45–60.

Hinkle, P. C., Kim, J. J., & Racker, E. (1972) *J. Biol. Chem. 247*, 1338–1339.

Hochman, J. H., Partridge, B., & Ferguson-Miller, S. (1981) *J. Biol. Chem. 256*, 8693–8698.

Kagawa, Y., & Racker, E. (1971) *J. Biol. Chem. 246*, 5477–5487.

Kuboyama, M., Yong, F. C., & King, T. E. (1972) *J. Biol. Chem. 247*, 6375–6383.

Massey, V. (1959) *Biochim. Biophys. Acta 34*, 255–256.

Mitchell, P. (1968) *Chemiosmotic Coupling and Energy Transduction*, Glynn Research Ltd., Bodmin, U.K.

Mitchell, P., & Moyle, J. (1983) *FEBS Lett. 151*, 167–178.

Nicholls, P., Hildebrandt, V., & Wrigglesworth, J. M. (1980) *Arch. Biochem. Biophys. 204*, 533–543.

Penefsky, H. S. (1979) *Methods Enzymol. 56*, 527–530.

Penttilä, T. (1983) *Eur. J. Biochem. 133*, 355–361.

Penttilä, T., & Wikström, M. (1981) in *Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria* (Palmieri, F., et al., Eds.) pp 71–80, Elsevier/North-Holland Biomedical Press, Amsterdam.

Pladziewicz, J. R., Meyer, T. M., Broomhead, J. A., & Taube, H. (1973) *Inorg. Chem. 12*, 639–643.

Pressman, B. C. (1976) *Annu. Rev. Biochem. 45*, 501–530.

Prochaska, L. J., Bisson, R., Capaldi, R. A., Steffens, G. C. M., & Buse, G. (1981) *Biochim. Biophys. Acta 637*, 360–373.

Reichardt, J. K. V., & Gibson, Q. H. (1982) *J. Biol. Chem. 257*, 9268–9270.

Reynafarje, B., Alexandre, A., Davies, P., & Lehninger, A. L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7218–7222.

Saraste, M., Penttilä, T., & Wikström, M. (1981) *Eur. J. Biochem. 115*, 261–268.

Schneider, D. L., Kagawa, Y., & Racker, E. (1971) *J. Biol. Chem. 247*, 4074–4079.

Scott, R. A., & Gray, H. B. (1980) *J. Am. Chem. Soc. 102*, 3219–3224.

Sigel, E., & Carafoli, E. (1978) *Eur. J. Biochem. 89*, 119–123.

Sone, N., Hamamoto, T., & Kagawa, Y. (1981) *J. Biol. Chem. 256*, 2873–2877.

Tarba, C. (1978) Ph.D. Thesis, Cornell University, Ithaca, NY.

Tarba, C. (1983) *Rev. Roum. Biochim. 20*, 151–159.

Wikström, M. K. F. (1972) *Biochim. Biophys. Acta 283*, 385–390.

Wikström, M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4051–4054.

Wikström, M. K. F., & Saari, H. T. (1977) *Biochim. Biophys. Acta 462*, 347–361.

Wikström, M., & Krab, K. (1979) *Biochim. Biophys. Acta 549*, 177–222.

Wilson, D. F., Lindsay, J. G., & Brocklehurst, E. S. (1972) *Biochim. Biophys. Acta 256*, 277–286.

Wilson, D. F., Erečinska, M., & Owen, C. S. (1976) *Arch. Biochem. Biophys. 175*, 160–173.

Wrigglesworth, J. M. (1978) *Proc. FEBS Meet. 45*, 95–103.

Wrigglesworth, J. M., & Nicholls, P. (1978) *FEBS Lett. 91*, 190–193.

Yonetani, T. (1966) *Biochem. Prep. 11*, 14–20.