Cytochrome *c* Oxidase Exhibits a Rapid Conformational Change upon Reduction of Cu_A: A Tryptophan Fluorescence Study†

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Received April 21, 1987; Revised Manuscript Received June 12, 1987

ABSTRACT: When cytochrome *c* oxidase is reduced, it undergoes a conformational change that shifts its tryptophan fluorescence maximum from 329 to 345 nm. Studies of ligand-bound, mixed-valence forms of the enzyme show that this conformational change is dependent on the redox state of the low-potential metal centers, cytochrome *a* and Cu_A. The intrinsic fluorescence of oxidized cytochrome *c* oxidase is not effectively quenched by Cs^+; however, marked quenching is observed for the reduced enzyme with a Stern–Volmer constant of 0.69. These observations, together with the significant red shift of the emission maximum, suggest that the emitting tryptophan residues are becoming more solvent accessible in the reduced enzyme. Stopped-flow spectra show that this conformational transition occurs rapidly upon reduction of the low-potential sites with a pseudo-first-order rate constant of $4.07 \pm 0.40\ s^{-1}$. The conformational change monitored by tryptophan fluorescence is suggested to be related to the previously proposed "open–closed" transition of cytochrome *c* oxidase. Reductive titration of the cyanide-inhibited enzyme with ferrocytochrome *c* shows a nonlinear response of the fluorescence shift to added electron equivalents. A theoretical treatment of the reduction of the two interacting sites of the cyanide-inhibited enzyme has been developed that gives the population of each redox state as a function of the total number of electrons accepted by the enzyme. This treatment depends on two parameters: the difference in redox potential between the two metals and the redox interaction between the redox centers. By use of literature values of these two parameters for the cyanide-inhibited enzyme, the expected behavior has been evaluated for three situations: (i) a conformational change induced by reduction of both cytochrome *a* and Cu_A (a two-electron process), (ii) a conformational change induced by reduction of cytochrome *a* (a one-electron process), and (iii) a conformational change induced by reduction of Cu_A (also a one-electron process). The results of this theoretical treatment strongly suggest that the current data, as well as independent data from other laboratories, are most consistent with a conformational change induced by reduction of the Cu_A center. The nature of this conformational change and its implication for proton pumping mechanisms of the enzyme are discussed.

Aerobic organisms have flourished because of the efficient means by which they utilize molecular oxygen to drive the biosynthesis of adenosine triphosphate (Lehninger, 1973). The reduction of dioxygen to water is catalyzed in these organisms by the terminal enzyme in the respiratory electron-transfer chain, cytochrome *c* oxidase. In mammals, cytochrome *c* oxidase spans the inner mitochondrial membrane, accepting electrons from ferrocytochrome *c* on the cytosolic side and donating them to dioxygen. The scalar protons consumed in this reaction are taken up from the matrix side of the membrane. Four metal cofactors are used in the electron-transfer reaction, two heme irons (cytochromes *a* and a_3) and two copper ions (Cu_A and Cu_B). Cytochrome *a* and Cu_A are located on the electron input side of the enzyme and serve as the primary electron acceptors from cytochrome *c*. Cytochrome a_3 and Cu_B together form the binuclear center for dioxygen binding (Wikström et al., 1981).

Apart from their role in electron transfer, at least one of these metal centers is associated with a redox-linked proton pumping activity in cytochrome *c* oxidase, translocating protons from the matrix to the cytosolic side of the membrane against a transmembrane proton gradient (Wikström et al., 1981). The energy derived in this way is used to drive the phosphorylation of adenosine diphosphate (ADP) to form adenosine triphosphate (ATP), the common energy currency of all organisms (Wikström et al., 1981). Work by Casey and Wikström has narrowed down the possible metal centers involved in proton pumping to the low-potential sites, cytochrome *a* and/or Cu_A (Wikström & Casey, 1985).

Over the years there has been mounting evidence that a large-scale conformational change of cytochrome *c* oxidase is associated with reduction of the two low-potential sites. Thus Cabral and Love (1972) showed via sedimentation studies that reduction of the enzyme resulted in a ca. 3% increase in the protein volume. The majority of this volume change was attributed to reduction of the low-potential sites via comparison of the fully reduced and CO mixed-valence (low-potential sites oxidized, high-potential sites reduced) complex. Chan and co-workers (Ellis et al., 1986; Wang et al., 1986) subsequently showed that the standard entropies of reduction were in fact quite large for both cytochrome *a* ($\Delta S^{\circ\prime} = -50.8$ eu) and Cu_A ($\Delta S^{\circ\prime} = -48.7$ eu) in the CO-inhibited enzyme, a result that would be consistent with relatively large protein conformational changes upon reduction of each of these sites. Yamamoto and Okunuki (1970) have shown that the reduced enzyme is more susceptible to digestion by proteinase than is the oxidized form. Circular dichroism spectroscopy has also revealed a large decrease in the positive ellipticity at 600 nm upon conversion of the fully reduced CO complex of cytochrome *c* oxidase to the mixed-valence form (Yong & King, 1970). This change was attributed to a protein conformational change upon ox-

† Contribution No. 7581 from the Arthur Amos Noyes Laboratory of Chemical Physics. This work was supported by Grant GM22432 from the National Institute of General Medical Sciences, U.S. Public Health Service.

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0006-2960/87/0426-7311$01.50/0

idation of the low-potential sites (Yong & King, 1970). A number of laboratories have shown that reduction of the low-potential sites leads to rapid exogenous ligand binding (Van Buuren et al., 1972; Jones et al., 1984; Jensen et al., 1984; Scholes & Malmström, 1986). These experiments indicate that a gross conformational change in the enzyme, upon reduction of the low-potential sites, results in "opening" of the oxygen binding site. For this reason this conformational change has been termed the "open–closed" transition.

Large-scale reorganizations of the protein matrix upon oxidoreduction are uncommon in electron-transfer proteins and in fact should be counterproductive to rapid electron transfer (Marcus & Sutin, 1985). This has led some workers to implicate the observed redox-linked conformational change in cytochrome *c* oxidase in the mechanism of transmembrane proton pumping by this enzyme (Jensen et al., 1984). If this is the case, a spectroscopic handle on such a conformational change would be quite important as it would allow one to monitor, in real time, the structural changes involved in cytochrome *c* oxidase proton pumping.

In this paper we report that tryptophan fluorescence spectroscopy can serve as such a probe of the low-potential-site redox-linked conformational change of cytochrome *c* oxidase. It will be shown that reduction of the low-potential sites of cytochrome *c* oxidase results in a conformational change that leads to solvent exposure of tryptophan residues which are otherwise buried in the oxidized state at least 30 Å away from the heme cofactors. This conformational change is manifested as a relatively large (1410 cm^{-1}) red shift of the tryptophan emission maximum, as well as a significantly increased level of collisional quenching by cesium chloride in the reduced enzyme. Stopped-flow fluorescence measurements show that this conformational change occurs rapidly upon reduction of the low-potential sites, on a time scale consistent with the open–closed conformational change reported by Scholes and Malmström (1986). Reductive titration of the cyanide-inhibited enzyme with ferrocytochrome *c* shows a nonlinear response of the fluorescence change to added electron equivalents. A theoretical treatment of these data suggests that the conformational change observed here is linked to reduction of the Cu_A center exclusively. The results are discussed in terms of possible mechanisms of proton pumping in cytochrome *c* oxidase.

Materials and Methods

Materials. Bovine heart cytochrome *c* oxidase was isolated by the method of Hartzell and Beinert (1974). The enzyme was solubilized in 0.05 M Hepes[1] buffer (pH 7.4) containing 0.5% Tween-20 (Sigma) and stored at −80 °C until just prior to use. Enzyme concentrations were determined spectrophotometrically by using a reduced minus oxidized $\Delta\epsilon$ value of 27 mM^{-1} cm^{-1} (for the wavelength pair 605–630 nm; Nicholls & Chanady, 1982). In a typical experiment the enzyme was thawed on ice and diluted with 0.5% lauryl maltoside (Calbiochem), 0.05 M Hepes, or potassium phosphate buffer (pH 7.4) to give an optical density at the excitation wavelength (270–290 nm) of between 0.70 and 0.75 (ca. 2–3 μM).

The oxidized and mixed-valence cyanide- (CN^-) bound and the fully reduced and mixed-valence carbon monoxide (CO) complexes of cytochrome *c* oxidase were prepared as previously described (Blair et al., 1986). Reduction of the metal centers was accomplished by addition of either sodium dithionite or ferrocytochrome *c* to the oxidase solution, which had previously been purged with N_2 for 15 min. The degree of reduction was determined spectrophotometrically both before and after acquisition of fluorescence data.

For quenching studies solid cesium chloride (Fisher) was added to resting cytochrome *c* oxidase to give the desired concentration of quencher, and the samples were incubated at 4 °C for 24 h before fluorescence measurements were performed.

Ferrocytochrome *c* was prepared by the reduction of ferricytochrome *c* (equine, Sigma type VI) with excess sodium dithionite (Aldrich). Residual dithionite was removed by column chromatography (Sephadex G-25) followed by extensive (>48 h) dialysis against 3000 volumes of buffer. The concentration of ferrocytochrome *c* was determined spectrophotometrically with an extinction coefficient of 29.5 mM^{-1} cm^{-1} at 550 nm (Sigma Chemical Co. catalog, 1986).

Experimental Methods. Steady-state fluorescence spectra were recorded on a SLM 4800 spectrofluorometer equipped with a SMC-210 monochromator controller and SE-480-485 electronics (SLM Instruments), which was interfaced to an IBM XT computer. The monochromator controller was calibrated daily. Excitation and emission monochromator slit widths were set at 8- and 4-nm resolution, respectively. All reported spectra were obtained with the emission polarizer set at 54.7° to minimize spectral anomalies due to stray light scattering (Lakowicz, 1983). Emission intensities were corrected for variations in instrument response by reference to a 3 g/L solution of rhodamine B in ethylene glycol (Lakowicz, 1983). Spectra were obtained at a scan speed of 1 nm/s, and each reported spectrum is the sum of 4 scans. The fluorescence spectra were corrected for inner filter effects by using the formula (Lakowicz, 1983)

$$F_{corr} = F_{obsd} \times 10^{(OD_{ex}+OD_{em})/2} \quad (1)$$

where F_{corr} is the corrected fluorescence intensity, F_{obsd} is the observed fluorescence intensity, and OD_{ex} and OD_{em} are the optical densities at the excitation and emission wavelengths, respectively.

Stopped-flow fluorescence was obtained with a Durrum stopped-flow apparatus with the photomultiplier tube mounted at 90° to the excitation beam path. The stopped-flow apparatus and computer-based data acquisition system were as previously described (Dunn et al., 1978, 1980). A pair of filters (Rollins 65.1620 and 65.1010) was placed between the sample and photomultiplier tube to form a transmittance (≥50%) window of 320–400 nm, with maximum transmittance at 350 nm. The excitation monochromator was set at 280 nm with a slit width of 4 nm. Samples of cytochrome *c* oxidase (2.53 μM) and ferrocytochrome *c* (12.95 μM) were degassed and loaded into the syringe barrels. The syringe plungers were actuated with nitrogen pressure, and the fluorescence intensity was monitored for either 1 or 5 s after mixing (dead time = 1 ms) with 1024 data points collected during the time interval. Fifteen consecutive scans were obtained. The data from each individual scan was fit to a single-exponential curve. The reported pseudo-first-order rate constant and $t_{1/2}$ values are the average values for 15 scans.

UV–vis absorption spectra (220–700 nm) were recorded for each sample before and after fluorescence spectra acquisition with a Beckman DU-7 HS scanning spectrophotometer.

Theoretical Analysis. Since cyanide inhibits the reduction of the cytochrome *c* oxidase high-potential metal centers (Wikström et al., 1981), a titration of the CN^--bound enzyme may be interpreted in terms of the equilibrium scheme shown in Figure 1. The equilibrium constants for the reduction of

[1] Abbreviations: Cs^+, cesium ion; $\Delta\epsilon$, extinction coefficient difference; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; UV, ultraviolet.

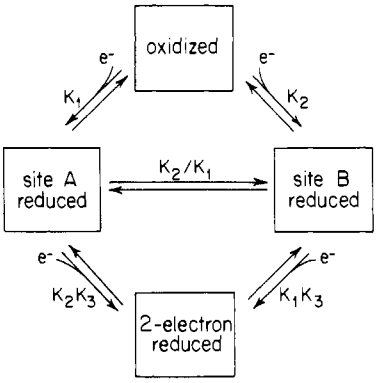

FIGURE 1: Equilibrium scheme for reduction of two interacting sites. K_1 and K_2 are the equilibrium constants for reduction of sites A and B, respectively, and K_3 is the contribution of the cooperativity to the equilibrium constant for reduction of the second site.

site A and site B from the oxidized enzyme are K_1 and K_2, respectively, while K_3 is the contribution of the cooperativity to the equilibrium constants for the reduction of site A or site B when the other site is already reduced. It should be noted that this scheme makes no mechanistic assumptions about how the equilibria are achieved. Whether site B is reduced directly from the oxidized enzyme or through site A depends on the relative rates of the processes, not on the equilibria. The relative populations of the four states of the enzyme can be expressed by the equations

$$n_A = K_1 n_O[R]$$
$$n_B = K_2 n_O[R] \quad (2)$$
$$n_R = K_2K_3 n_A[R] = K_1K_2K_3 n_O[R]^2$$

where n_O, n_A, n_B, and n_R are the fractional populations of oxidized, site A reduced, site B reduced, and two-electron-reduced enzyme, respectively, and [R] is the concentration of the reductant. By definition $n_O + n_A + n_B + n_R = 1$; therefore

$$n_O(1 + K_1[R] + K_2[R] + K_1K_2K_3[R]^2) = 1 \quad (3)$$

Using the substitution $x = K_1[R]$ to eliminate all constants that are specific to a particular reductant yields

$$n_O = 1/\{(K_2/K_1)K_3x^2 + [1 + (K_2/K_1)]x + 1\} \quad (4)$$

Combining this equation with the equilibrium expressions gives

$$n_A = x/\{(K_2/K_1)K_3x^2 + [1 + (K_2/K_1)]x + 1\} \quad (5)$$

$$n_B = (K_2/K_1)x/\{(K_2/K_1)K_3x^2 + [1 + (K_2/K_1)]x + 1\} \quad (6)$$

$$n_R = (K_2/K_1)K_3x^2/\{(K_2/K_1)K_3x^2 + [1 + (K_2/K_1)]x + 1\} \quad (7)$$

The number of electron equivalents transferred per molecule of enzyme, N, is

$$N = n_A + n_B + 2n_R \quad (8)$$

Substituting eq 5–7 for n_A, n_B, and n_R, we obtain

$$N = \frac{2(K_2/K_1)K_3x^2 + [(K_2/K_1) + 1]x}{(K_2/K_1)K_3x^2 + [1 + (K_2/K_1)]x + 1} \quad (9)$$

Plots of the state populations vs N were obtained by varying x and calculating the resultant n_A, n_B, n_R, and N. However, (9) is only a quadratic equation, so x can be eliminated and the populations can be written as explicit functions of N.

Equations 4–7 and 9 depend only on (K_2/K_1) and K_3, which were calculated from the literature values for $\Delta E^{\circ\prime}_{cyt\,a\text{-}Cu_A}$ and $\Delta E^{\circ\prime}_{cooperative}$ by using the Nernst equation for $T = 25$ °C: $K = 10^{\Delta E^{\circ\prime}/0.059}$. $\Delta E^{\circ\prime}_{cyt\,a\text{-}Cu_A}$ is the difference in reduction potential between cytochrome a and Cu_A in the fully oxidized cyanide-inhibited enzyme [the experimental value of $\Delta E^{\circ\prime}_{cyt\,a\text{-}Cu_A}$ was taken from Goodman (1984)], and $\Delta E^{\circ\prime}_{cooperative}$ is the change in reduction potential of cytochrome a or Cu_A upon reduction of the other metal center [the experimental value of $\Delta E^{\circ\prime}_{cooperative}$ for the cyanide-inhibited enzyme was taken from Ellis (1986)].

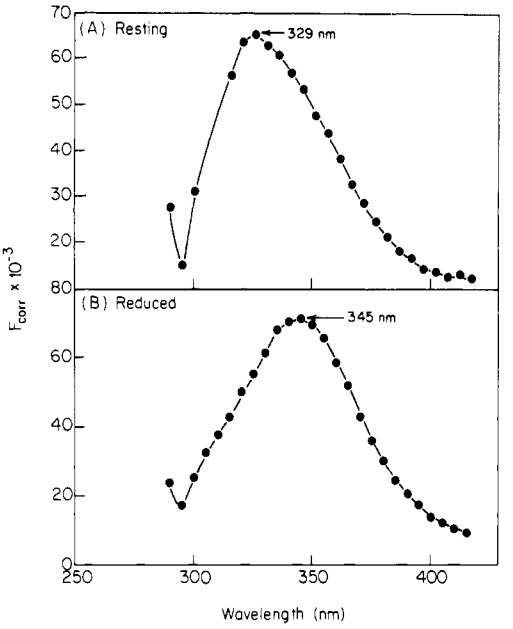

FIGURE 2: Corrected fluorescence spectra of (A) resting and (B) fully reduced cytochrome c oxidase (1.96 μM) obtained with 280-nm excitation. See text for further details.

RESULTS

Figure 2 shows the corrected steady-state fluorescence spectra, excited at 280 nm, of resting (all metal centers oxidized) and fully reduced cytochrome c oxidase. Similar results were obtained with 270- and 290-nm excitation for both forms of the enzyme, indicating that all of the fluorescence intensity can be attributed to tryptophan (Hill et al., 1986). As first reported by Hill et al. (1986), the resting enzyme exhibits an emission maximum at 329 nm, which is significantly blue-shifted relative to typical solvent-exposed tryptophan residues on protein surfaces. These workers noted, however, that a similar blue shift is observed for free indole in 0.5% lauryl maltoside solution [the detergent system used to solubilize cytochrome c oxidase in the present study, as well as in the work of Hill et al. (1986)]. However, to our surprise, reduction of the metal centers leads to a 16-nm (1410 cm^{-1}) red shift of the tryptophan maximum relative to the resting enzyme. Since the quantum yields and bandwidths at half-maximum are very similar in the resting and reduced enzymes, it is unlikely that different sets of tryptophan residues are being monitored in the two spectra. We surmise that the emitting tryptophans have undergone a change in environment which causes their composite fluorescence spectrum to appear more like that of an exposed residue.

Since the heme cofactors of cytochrome c oxidase should be quite effective in quenching emission from tryptophan residues in close spatial proximity, Hill et al. (1986) concluded that the fluorescence spectrum must arise from a relatively small number of tryptophans at an average distance of 30 Å from the heme groups, on the basis of the critical Förster distance estimated for tryptophan–heme resonance energy transfer. The emitting tryptophans must also be a considerable distance from the cytochrome c binding site of the enzyme

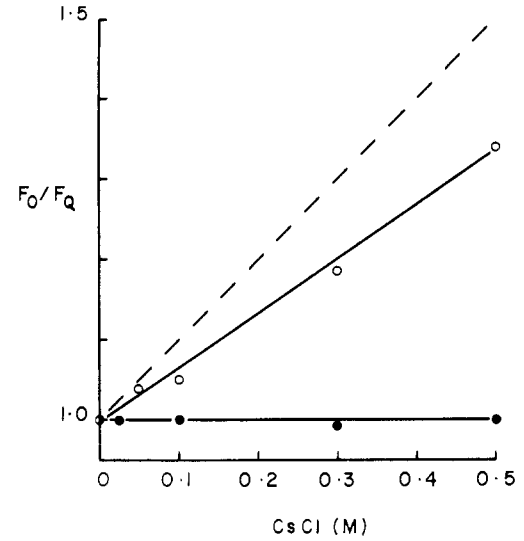

FIGURE 3: Stern–Volmer plot of CsCl quenching of resting (filled circles) and fully reduced (open circles) cytochrome *c* oxidase (2.00 μM). The dashed line represents the expected behavior for free indole in 0.5% lauryl maltoside solution [from Hill et al. (1986)].

because of the lack of emission quenching in the 1:1 cytochrome *c*:cytochrome *c* oxidase complex (Hill et al., 1986).

A red shift of the fluorescence maximum is usually attributed to an increase in polarity of the medium surrounding the fluorophore (Lakowicz, 1983). Such a polarity change could be the result of changes in charged amino acid side chains within the interior of the protein or a change in solvent accessibility of the fluorophores. Hill et al. (1986) showed that the emitting tryptophans of resting-state cytochrome *c* oxidase were not quenched by cesium ion, although indole in lauryl maltoside solution was effectively quenched. This result would suggest that the emitting tryptophans are buried in the hydrophobic interior of the enzyme in the resting state. We have extended the Cs^+ quenching studies of Hill et al. (1986) to include the reduced state of the enzyme (Figure 3). In agreement with Hill et al., we find no quenching of the tryptophan fluorescence in resting-state cytochrome *c* oxidase at Cs^+ concentrations as high as 0.5 M. In contrast, the reduced enzyme shows a significantly greater degree of quenching by cesium, with a Stern–Volmer constant, K_{SV}, of 0.69, which is close to the value found for free indole in lauryl maltoside solution (K_{SV} = 1.0, represented by the dashed line in Figure 3) reported by Hill et al. (1986). These data suggest that the red-shifted emission maximum observed in reduced cytochrome *c* oxidase reflects an increased solvent exposure of the emitting residues rather than localized changes in charged amino acid residues.

We have attempted to address the issue of whether the redox-linked fluorescence changes seen here are associated with the reduction of a subset of the enzyme's metal centers. Toward this end we have examined a variety of mixed-valence states of the enzyme obtained by exogenous ligand binding. Binding of CN^- to the oxygen binding site locks the high-potential metal centers in their oxidized states but allows the low-potential sites to be reduced. CO binds to the fully reduced enzyme; however, the low-potential sites can be oxidized while the high potential sites remain reduced. The results of these studies are summarized in Table I. Consistently, we find that the tryptophan fluorescence is unaffected by ligand binding or oxidoreduction at the oxygen binding site. Instead, the tryptophan fluorescence is correlated with the oxidation state of the low-potential sites. These data argue that the conformational change responsible for the tryptophan fluorescence

Table I: Tryptophan Fluorescence Maxima for Various Forms of Cytochrome *c* Oxidase in 0.5% Lauryl Maltoside Solution

state	high-potential sites	low-potential sites	λ_{max} (nm)
resting	oxidized	oxidized	329
428-nm form[a]	oxidized	oxidized	329
CN bound	oxidized	oxidized	327
CN mixed valence	oxidized	reduced	341
CO mixed valence	reduced	oxidized	329
reduced CO	reduced	reduced	345
reduced	reduced	reduced	345

[a] The 428-nm form is prepared by air reoxidation of the reduced enzyme and is believed to be a heterogeneous state containing pulsed, compound C, and ferryl forms of the enzyme (Orii & King, 1976).

change is triggered by reduction of either cytochrome *a* or Cu_A or both.

In order to further pinpoint the redox center responsible for this redox-linked conformational change, we performed a reductive titration of the CN^--inhibited enzyme (Scholes & Malmström, 1986). Reductive equivalents were delivered to the enzyme via ferrocytochrome *c*, and the corrected fluorescence intensity was measured at 350 nm. The number of equivalents actually transferred was determined by monitoring oxidation of the added cytochrome *c* using absorption spectroscopy (Scholes & Malmström, 1986). A measure of the fluorescence change can be taken to be

$$\Delta F = (F_N - F_0)/(F - F_0) \qquad (10)$$

where F_N is the corrected fluorescence intensity after addition of N equivalents of ferrocytochrome *c*, F_0 is the corrected fluorescence intensity of fully oxidized CN^--bound oxidase (i.e., zero equivalents added), and F is the corrected fluorescence intensity of the mixed-valence CN^- complex prepared by reduction of the oxidized CN^--bound enzyme with a slight excess of dithionite.

In a similar fashion Scholes and Malmström (1986) monitored the initial velocity (v) of CN^- inhibition of the enzyme as a function of added electron equivalents (N). Their data were best fit by the equation $v = 2.2 \times 10^{-4} + 2.5 \times 10^{-4}N^2$. Using this equation, we have calculated v for various levels of reduction (N) and have converted these data to a form similar to ours by using the equation

$$\Delta v = (v_N - v_0)/(v - v_0) \qquad (11)$$

where v_N and v_0 are defined similarly to F_N and F_0 of eq 10 and v is the initial velocity calculated from Scholes and Malmström's equation for N = 2.0.

We interpreted the measured fluorescence change as a sum of fluorescence changes due to each of the reduced states

$$\Delta F = n_{Cu_A}\Delta F_{Cu_A} + n_{cyta}\Delta F_{cyta} + n_R\Delta F_R \qquad (12)$$

where ΔF_i is the fluorescence change that would be measured if the enzyme were entirely in state i and n_i is the population of the enzyme in state i. The solid curve in Figure 4 is the predicted behavior for a ΔF that is entirely dependent on reduction of Cu_A ($\Delta F_R = \Delta F_{Cu_A} = 1$; $\Delta F_{cyta} = 0$) when the reduction potential for Cu_A is 48 mV lower than that of cytochrome *a* (Goodman, 1984) and there is a 40-mV anticooperative interaction between the potentials of these two sites (Wang et al., 1986; Ellis, 1986). The dashed-dotted curve in Figure 4 represents the predicted behavior for a ΔF that is entirely dependent on the reduction of cytochrome *a* ($\Delta F_R = \Delta F_{cyta} = 1$; $\Delta F_{Cu_A} = 0$), while the dashed curve shows the expected behavior of a ΔF that depends on the reduction of *both* cytochrome *a* and Cu_A ($\Delta F_R = 1$; $\Delta F_{Cu_A} = \Delta F_{cyta} = 0$) by use of the same values for the reduction potential difference

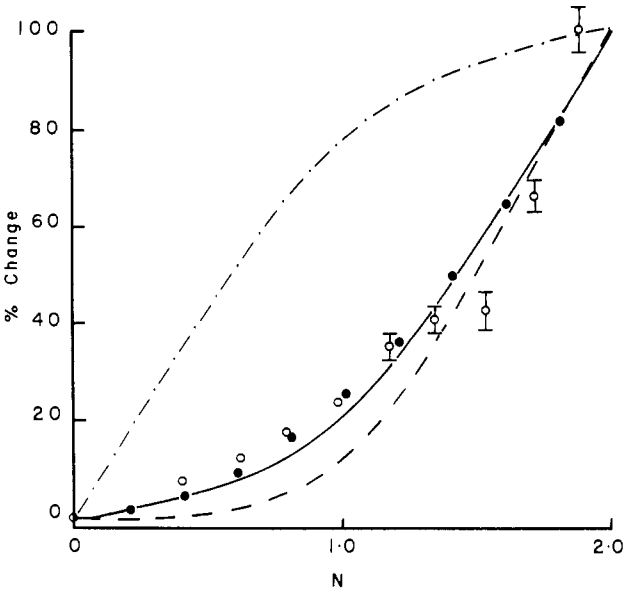

FIGURE 4: Plot of percent change vs electron equivalents accepted (N) from ferrocytochrome c by cyanide-inhibited cytochrome c oxidase. The open circles represent the percent change in fluorescence intensity at 350 nm (for the points without error bars the estimated error is less than or equal to the diameter of the circle representing the data point). The filled circles represent the percent change in initial velocity of cyanide inhibition [data adapted from Scholes and Malmström (1986)]. The solid curve is the theoretically predicted behavior for a one-electron process at the Cu_A site, the dashed-dotted curve is the theoretically predicted behavior for a one-electron process at cytochrome a, and the dashed line is the theoretically predicted behavior for a two-electron process at cytochrome a and Cu_A.

and cooperativity as above. Figure 4 also includes the results of our reductive titration (open circles) as well as the data adapted from Scholes and Malmström (1986) (filled circles). Both data sets are best described by the Cu_A-dependent process, particularly in the region below $N = 1$ where the two-electron process shows virtually no response to added electron equivalents. The theoretical curves in Figure 4 are relatively insensitive to changes in the strength of anticooperativity, although values of ca. 20 mV fit the experimental data best. Varying the relative reduction potentials of cytochrome a and Cu_A had a larger effect, but in all cases reduction of Cu_A correlated better with the experimental data than did two-electron reduction or cytochrome a reduction.

In order for the conformational change monitored by tryptophan fluorescence to be of any mechanistic significance, it must occur rapidly upon reduction of the low-potential sites, on a time scale consistent with enzyme turnover. We have addressed this question by using stopped-flow spectroscopy to study the time course of the fluorescence change. A typical stopped-flow scan is shown in Figure 5. The solid curve through the data corresponds to the best-fit curve for a single-exponential process. Fifteen such scans were obtained, yielding an average pseudo-first-order rate constant of 4.07 $\pm$ 0.40 s^{-1} (or an average $t_{1/2}$ of 0.17 $\pm$ 0.02 s). Thus, the conformational change observed here is at least as fast as the open–closed transition (rate constant = 1.0 s^{-1}, $t_{1/2}$ = 0.69 s) observed by Scholes and Malmström (1986). Since the open–closed transition can occur on a time scale consistent with enzyme turnover (Jones et al., 1984), our results imply by analogy that the conformational change seen here may also be accessible during enzyme turnover.

Discussion

The majority of reports of conformational changes in cytochrome c oxidase are based on indirect evidence from study

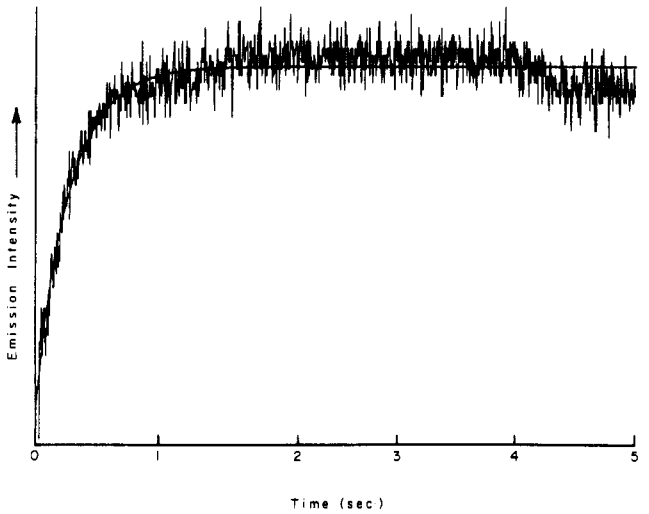

FIGURE 5: Typical stopped-flow fluorescence trace of reduction of cytochrome c oxidase (2.53 μM) by ferrocytochrome c (12.95 μM). The darker line through the trace represents the best single-exponential fit to the data.

of the behavior of the metal cofactors. The results reported here provide the first direct spectroscopic evidence for changes in the protein matrix upon oxidoreduction at the low-potential metal centers. The time scale of the conformational change monitored here and its reductive titration behavior are quite similar to the results obtained by Scholes and Malmström (1986) for the open–closed transition of cytochrome c oxidase. It seems likely that both groups are observing manifestations of the same conformational change.

The reductive titration data of Scholes and Malmström (1986) were originally interpreted as implying a process that was dependent on reduction of *both* low-potential metal centers. These authors argued that any process dependent on reduction of a single metal center should yield a linear relationship between added reductive equivalents and the observed conformational change, even in the presence of moderate anticooperativity between the low-potential metal sites. This interpretation assumes that the redox potentials of cytochrome a and Cu_A are identical in the CN^--inhibited cytochrome c oxidase (Antalis & Palmer, 1982). It is well-known, however, that the reduction potential of cytochrome a is ca. 48 mV above that of Cu_A in this form of the enzyme (Goodman, 1984). With a redox potential difference of this magnitude between the low-potential centers, one would not expect the conformational change to respond linearly for either a one-electron or a two-electron process. Thus three possibilities exist: (i) the conformational change is triggered by reduction of cytochrome a, (ii) the conformational change is triggered by reduction of Cu_A, or (iii) the conformational change is triggered only after reduction of both cytochrome a and Cu_A. Since the redox potential of cytochrome a is higher than that of Cu_A, we can immediately exclude the first possibility from the shape of the plots in Figure 4. Of the two possibilities remaining, our theoretical treatment of the data in Figure 4 clearly indicates that the one-electron process describes the data far better than does the two-electron process. We thus conclude that the observed conformational change is triggered by reduction of the Cu_A site.

Gelles et al. (1987) have presented a detailed theoretical treatment of proton pumping in cytochrome c oxidase in which they argue in favor of Cu_A as the site of the redox link. A ligand substitution reaction at the Cu_A site upon electron input was proposed as the structural basis for redox-linked proton pumping in this protein. The recent demonstrations that

modification of the Cu_A center is correlated with disruption of proton pumping activity in cytochrome *c* oxidase (Li et al., 1987; Nilsson et al., 1987) lend strong support to the model presented by Gelles et al. (1987). The results presented here are likewise consistent with this model. A rearrangement of the ligands about Cu_A could be envisioned to cause a conformational change that is felt at some distance from the metal center, in much the same way that oxygen binding to deoxyhemoglobin causes changes in hydrogen bonding at the distal subunit interfaces of this protein (Dickerson & Geis, 1983). While such gross conformational changes are not necessarily required for proton pumping proteins (Gelles et al., 1987), they are not inconsistent with such activities. In fact, theoretical models of proton pumping in cytochrome *c* oxidase have been put forth in which a protein conformational change is specifically implicated (Wilkström, 1978).

In summary, we have presented evidence that reduction of Cu_A results in a large-scale conformational change of cytochrome *c* oxidase which affects the solvent accessibility of tryptophan residues at some distance from the metal center. This conformational change is suggested to be analogous to the open–closed transition previously reported. It may be that this conformational change is involved in the proton pumping activity of cytochrome *c* oxidase. In this regard it will be interesting to see if this conformational change can be observed in cytochrome *c* oxidase reconstituted into phospholipid vesicles and whether it is affected by applied potential gradients across the membrane. Experiments to address these issues are under way in our laboratory.

ACKNOWLEDGMENTS

We thank Professor Michael F. Dunn for the use of a Durrum stopped-flow apparatus and Thomas Nilsson, Joel E. Morgan, and N. Elise Gabriel for helpful discussions.

Registry No. Cu, 7440-50-8; cytochrome *c* oxidase, 9001-16-5.

REFERENCES

Antalis, T. M., & Palmer, G. (1982) *J. Biol. Chem. 257*, 6194.

Blair, D. F., Ellis, W. R., Wang, H., Gray, H. B., & Chan, S. I. (1986) *J. Biol. Chem. 261*, 11524.

Cabral, F., & Love, B. (1972) *Biochim. Biophys. Acta 283*, 181.

Dickerson, R. E., & Geis, I. (1983) *Hemoglobin*, Benjamin/Cummings, Menlo Park, CA.

Dunn, M. F., Bernhard, S. A., Anderson, D., Copeland, A., Morris, R. G., & Roque, J. P. (1979) *Biochemistry 18*, 2346.

Dunn, M. F., Pattison, S. E., Storm, M. C., & Quiel, E. (1980) *Biochemistry 19*, 718.

Ellis, W. R. (1986) Ph.D. Thesis, California Institute of Technology, Pasadena, CA.

Ellis, W. R., Wang, H., Blair, D. F., Gray, H. B., & Chan, S. I. (1986) *Biochemistry 25*, 161.

Gelles, J., Blair, D. F., & Chan, S. I. (1987) *Biochim. Biophys. Acta (Rev.) 853*, 205.

Goodman, G. (1984) *J. Biol. Chem. 259*, 15094.

Hartzell, C. R., & Beinert, H. (1974) *Biochim. Biophys. Acta 368*, 318.

Hill, B. C., Horowitz, P. M., & Robinson, N. C. (1986) *Biochemistry 25*, 2287.

Jensen, P., Wilson, M. T., Aasa, R., & Malmström, B. G. (1984) *Biochem. J. 224*, 829.

Jones, M. G., Bickar, D., Wilson, M. T., Brunori, M., Colosimo, A., & Sarti, P. (1984) *Biochem. J. 220*, 57.

Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, pp 341–381, Plenum, New York.

Lehninger, A. L. (1973) *Bioenergetics*, Benjamin/Cummings, Menlo Park, CA.

Li, P. M., Nilsson, T., Copeland, R. A., & Chan, S. I. (1987) *Biochemistry* (submitted for publication).

Marcus, R. A., & Sutin, N. (1985) *Biochim. Biophys. Acta 811*, 265.

Nicholls, P., & Chanady, G. A. (1982) *Biochem. J. 203*, 541.

Nilsson, T., Gelles, J., Li, P. M., & Chan, S. I. (1987) *Biochemistry* (in press).

Orii, Y., & King, T. E. (1976) *J. Biol. Chem. 251*, 7487.

Scholes, C. P., & Malmström, B. G. (1986) *FEBS Lett. 198*, 125.

Van Buuren, K. J. H., Zuurendonk, P. F., Van Gelder, B. F., & Muijsers, A. O. (1972) *Biochim. Biophys. Acta 256*, 243.

Wang, H., Blair, D. F., Ellis, W. R., Gray, H. B., & Chan, S. I. (1986) *Biochemistry 25*, 167.

Wikström, M. (1978) in *The Proton and Calcium Pumps* (Azzone, G. F., et al., Eds.) pp 215–226, Elsevier/North-Holland, New York.

Wikström, M., & Casey, R. P. (1985) *J. Inorg. Biochem. 23*, 327.

Wikström, M., Krab, K., & Saraste, M. (1981) *Cytochrome Oxidase, A Synthesis*, Academic, New York.

Yamamoto, T., & Okunuki, K. (1970) *J. Biochem. (Tokyo) 67*, 505.

Yong, F. C., & King, T. E. (1970) *Biochem. Biophys. Res. Commun. 40*, 1445.