Characterization of Reductant-Induced, Tryptophan Fluorescence Changes in Cytochrome Oxidase†

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ABSTRACT: We have measured the steady-state tryptophan fluorescence spectrum of cytochrome oxidase in its oxidized and fully reduced states. Reduction of the oxidized enzyme by sodium dithionite causes an apparent shift in the fluorescence emission maximum from 328 nm, in the oxidized enzyme, to 348 nm, in the reduced enzyme. This spectroscopic change has been observed previously and assigned to a redox-linked, conformational change in cytochrome oxidase [Copeland, R. A., Smith, P. A., & Chan, S. I. (1987) *Biochemistry 26*, 7311–7316]. When dithionite-reduced enzyme sits in an open cuvette, the enzyme returns to the oxidized state, and the fluorescence maximum shifts back to 328 nm. However, the time course of the fluorescence change does not follow the redox state of the enzyme, monitored spectrophotometrically at 445, 605, and 820 nm, but follows the disappearance of dithionite, which absorbs at 315 nm. Moreover, when the fluorescence emission spectrum of the dithionite-reduced enzyme is corrected for the absorbance due to dithionite, the fluorescence maximum is found 2 nm blue shifted, relative to that of the oxidized enzyme, at 326 nm. This dithionite-induced, red-shifted steady-state tryptophan fluorescence is also seen with the non-heme-containing enzyme carboxypeptidase A. The tryptophan emission spectrum of untreated carboxypeptidase A is at 332 nm, whereas in the presence of dithionite the emission spectrum of carboxypeptidase A is at 350 nm. When corrected for the absorbance of dithionite, the tryptophan emission maximum is at 332 nm. We have also used the photoreductant 3,10-dimethyl-5-deazaisoalloxazine (deazaflavin) to reduce cytochrome oxidase. Only catalytic amounts of deazaflavin are required, and the absorbance of this species in the region of the tryptophan emission is small. When the oxidase is reduced with deazaflavin, the uncorrected emission is at 328 nm, and the corrected spectrum is at 324 nm. We conclude that the 18-nm red shift of the tryptophan emission spectrum that is reported to occur when cytochrome oxidase is reduced, and ascribed specifically to the reduction of Cu_A [Copeland, R. A., Smith, P. A., & Chan, S. I. (1987) *Biochemistry 26*, 7311–7316; Copeland, R. A., Smith, P. A., & Chan, S. I. (1988) *Biochemistry 27*, 3552–3555], is due to the strong absorbance of dithionite in the region of the tryptophan emission spectrum. Our findings are presented as evidence against redox-linked, large-scale conformational changes within the cytochrome oxidase complex as possible intermediate steps in energy transduction.

Cytochrome oxidase catalyzes electron transfer from cytochrome *c* to oxygen and acts as a biological energy transducer by conserving some of the free energy of this exergonic redox reaction in an electrochemical ion gradient (Krab & Wikström, 1987). The ion gradient generated by cytochrome oxidase is partially composed of a transmembrane concentration gradient of protons. The mechanism whereby cytochrome oxidase links the free energy of electron transfer to transmembrane proton movement has not been determined. Direct roles in proton translocation have been suggested, variously, for cytochrome *a* (Callahan & Babcock, 1983), Cu_A (Gelles et al., 1987), and the binuclear cytochrome a_3–Cu_B center (Chance, 1981). In addition, protein conformational states, linked to the redox state of the oxidase, have been proposed as intermediates in the energy transduction process (Brzezinski & Malmström, 1987).

Cytochrome oxidase has been found to have intrinsic fluorescence arising from tryptophan residues of the protein complex (Hill et al., 1986). We have used this property to monitor the enzyme's conformation when exposed to denaturing conditions (Hill et al., 1988). Tryptophan fluorescence should prove useful in determining to what extent the oxidase's global conformational state is dependent upon the redox or ligation state of the enzyme's metal centers. In this paper we report the changes observed in the steady-state tryptophan fluorescence spectrum of cytochrome oxidase when it is reduced by sodium dithionite or the photochemical reductant deazaflavin.[1] In agreement with Copeland et al. (1987), we find a tryptophan emission spectrum shifted to the red by 20 nm when the enzyme is reduced by dithionite. This spectral change has been interpreted as indicating a large-scale, redox-linked conformational change in cytochrome oxidase with significant implications for the enzyme's mechanism of energy transduction. However, we find that when the observed emission spectrum of the dithionite-reduced enzyme is corrected for the absorbance of dithionite, or the enzyme is reduced by the photoreductant deazaflavin, the spectrum is very close to that of the oxidized enzyme. The conformational changes induced by redox turnover of the electron-carrying centers of the oxidase are not large in scale and argue against a long-range conformational hypothesis for electron–proton coupling in cytochrome oxidase.

Materials and Methods

Cytochrome oxidase was isolated by the method of Kuboyama et al. (1972) from beef heart, submitochondrial particles prepared according to Yonetani (1961). Cytochrome oxidase

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[1] Abbreviations: deazaflavin, 3,10-dimethyl-5-deazaisoalloxazine; EDTA, ethylenediaminetetraacetate.

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prepared by the method of Hartzell and Beinert (1974) was the kind gift of Dr. G. Palmer (Rice University). Stock solutions of sodium dithionite (Fisher) were prepared fresh daily in 100 mM phosphate buffer, pH 7.35, and were kept under an atmosphere of N_2. 3,10-Dimethyl-5-deazaisoalloxazine (deazaflavin) was a kind gift from Dr. Vincent Massey (University of Michigan). Deazaflavin was prepared in a 1 mM stock solution in dimethylformamide and used at a concentration of 6.7 μM. Carboxypeptidase A and lauryl maltoside were obtained from Boehringer Mannheim.

Cytochrome oxidase samples were prepared in buffer containing 20 mM Tris, 1 mM EDTA, 0.1 M NaCl, and 1 mg/mL lauryl maltoside, pH 7.8. Mixed-valence, CO-bound cytochrome oxidase was prepared by incubation of the resting enzyme under an atmosphere of CO for 20 h at room temperature. The mixed-valence species was taken to full reduction by (i) the addition of microliter aliquots from a 100 mM stock solution of sodium dithionite or (ii) photoreduction with deazaflavin and EDTA. Deazaflavin and 15 mM EDTA were added to enzyme samples that were to be photoreduced prior to the addition of CO. Photoreduction was achieved by irradiation for 1 min with the light from a Kodak slide projector.

Carboxypeptidase A was dialyzed against water to remove toluene, and samples were subsequently prepared in the same buffer used for cytochrome oxidase. Spectra and corrections were obtained identically with those for cytochrome oxidase samples.

Absorbance spectra were taken on a Shimadzu UV-160 UV–visible recording spectrophotometer. Fluorescence spectra were obtained on a Perkin-Elmer fluorescence spectrophotometer, Model MPF-44B. Fluorescence emission spectra were obtained by exciting at 280 nm and recording between 300 and 400 nm. Observed fluorescence spectra were corrected according to eq 1, where F_{obs} is the fluorescence intensity

$$F_{cor} = F_{obs} \text{ antilog } [(A_{ex} + A_{em})/2] \quad (1)$$

observed at a specific wavelength, A_{em} is the absorbance reading at that particular emission wavelength, and A_{ex} is the absorbance value at the excitation wavelength (Lakowicz, 1983). In the corrected spectra reported here, absorbance values and observed fluorescence intensities were used to obtain the corrected fluorescence intensity at 2-nm intervals from 300 to 400 nm.

Absorption spectra were obtained prior to and subsequent to fluorescence emission spectra acquisition to ensure that no changes had occurred in the absorption spectrum. A maximum absorbance change of 2% was seen over the time taken to obtain fluorescence spectra.

Results

Corrected and uncorrected fluorescence emission spectra of cytochrome oxidase are shown in Figure 1. The resting form of the enzyme (Figure 1A) has an emission maximum at 328 nm, as previously reported (Hill et al., 1986; Copeland et al., 1987). The fully reduced enzyme, with dithionite as reductant, has an uncorrected fluorescence emission maximum at 348 nm, in agreement with the observation made by Copeland et al. (1987). The corrected fluorescence emission maximum of the fully reduced oxidase is at 326 nm, blue shifted by 2 nm as compared to that of the resting enzyme.

In Figure 1B the absorption spectra of resting and fully reduced cytochrome oxidase are shown. The Soret bands of our resting and fully reduced oxidase samples are at 417 and 443.5 nm, respectively. In panel b of Figure 1B the absorbance spectrum of the reduced sample is presented on an appropriate

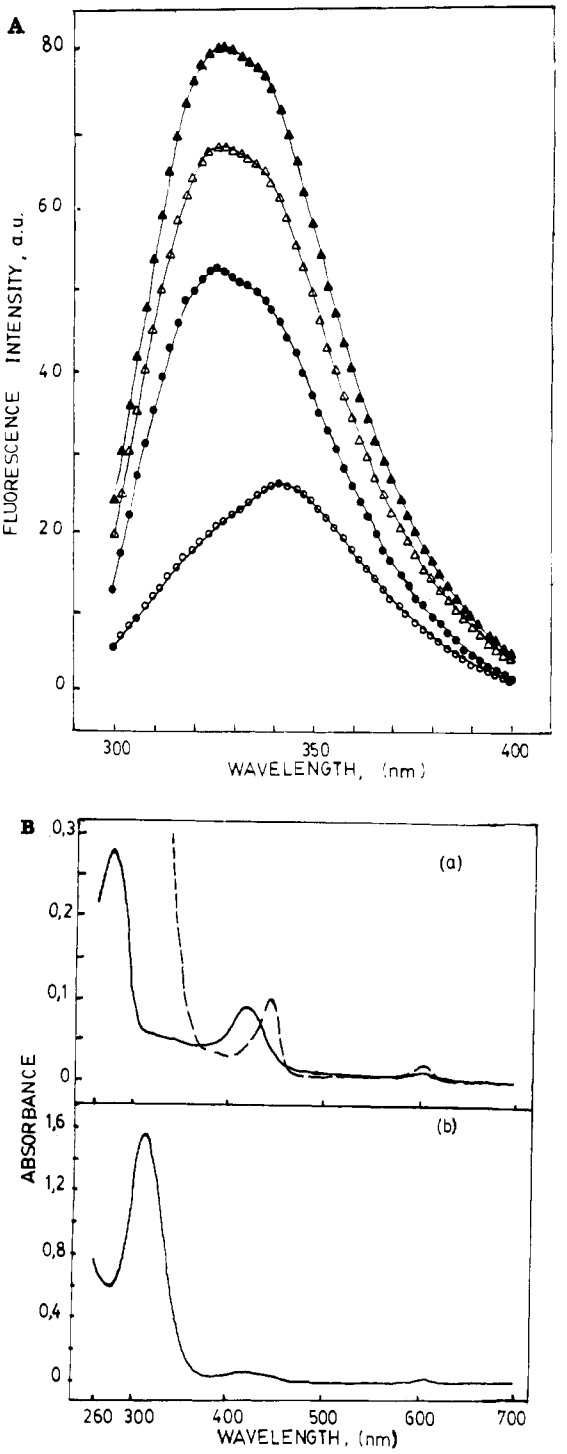

FIGURE 1: (A) Tryptophan fluorescence spectra of resting, oxidized and dithionite-reduced cytochrome oxidase. Corrected (solid) and uncorrected (open) fluorescence spectra of resting (▲, △) and fully reduced (●, ○) cytochrome oxidase (0.59 μM). Fluorescence spectra were obtained with 280-nm excitation and 10-nm band-pass on the excitation and emission monochromators. (B) Absorbance spectra in the UV and visible regions of cytochrome oxidase in the absence and presence of sodium dithionite. (a) Absolute absorbance spectra of cytochrome oxidase (0.59 μM) in the resting, oxidized (—) and dithionite-reduced (– –) states. Dithionite was added to a final concentration of 1 mM from a 100 mM stock solution. (b) The absorbance spectrum of sodium dithionite. Same sample as in (a) with the absorbance scale adjusted to show the peak due to dithionite.

scale to show the absorbance band due to dithionite. We observe an absorbance band with a maximum at 315 nm. Upon introduction of oxygen to the dithionite-reduced sample, the 315-nm peak disappears, and the Soret band of the enzyme is observed at 425 nm. The fluorescence emission spectrum

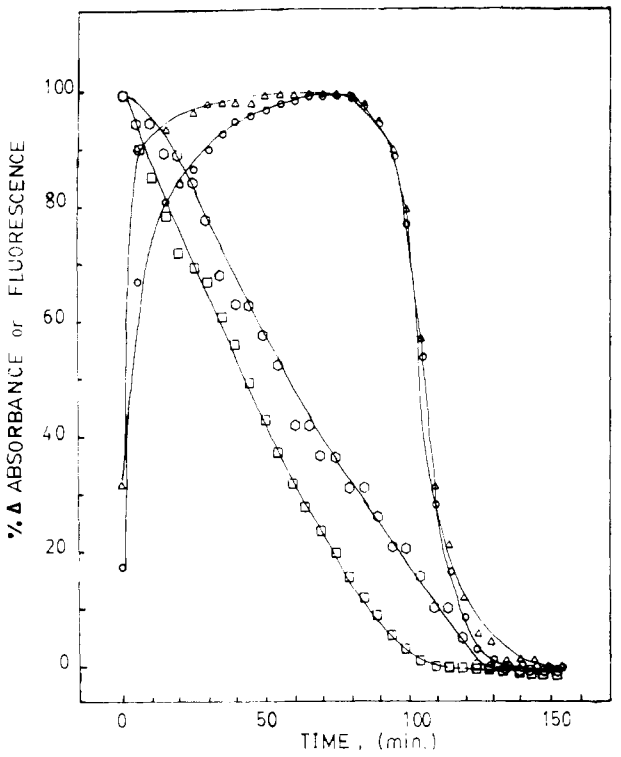

FIGURE 2: Time course of the reaction of sodium dithionite with cytochrome oxidase (3.4 μM) in an open cuvette. The absorbance was monitored at 315 (□), 445 (○), and 605 nm (△). The change in fluorescence emission shift (○) is reported as a percentage of the maximum wavelength shift observed, which was 20 nm. The dithionite concentration at the beginning of the experiment was 1 mM.

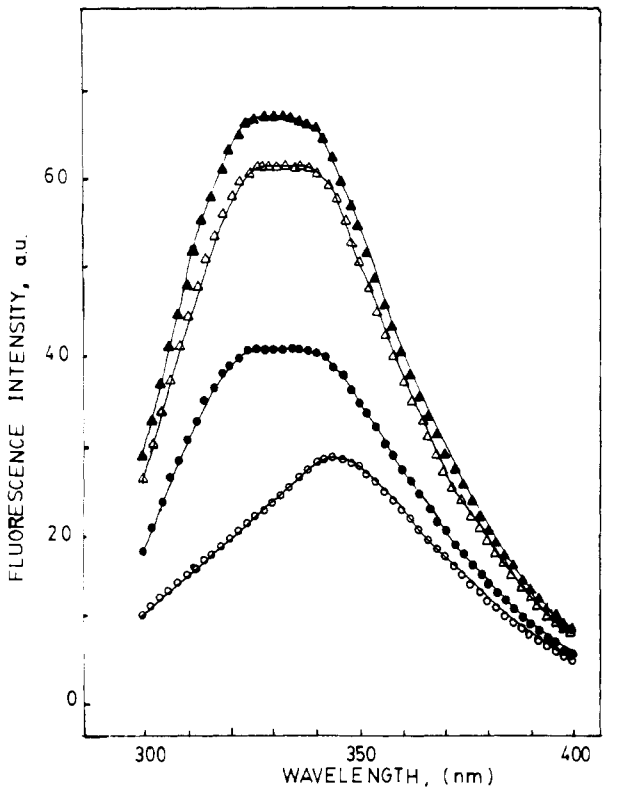

FIGURE 3: Effect of dithionite on the tryptophan emission spectrum of carboxypeptidase A. Corrected (solid) and uncorrected (open) fluorescence emission spectra of carboxypeptidase A in the presence (●, ○) and absence (▲, △) of dithionite. The dithionite concentration was 1 mM, and the instrumental conditions were the same as those outlined in the legend to Figure 1.

shifts to 348 nm in the dithionite-reduced sample and returns to 328 nm upon reoxidation.

A time course of the changes in the dithionite levels, the oxidase redox state, and the fluorescence emission maximum is shown in Figure 2 following the addition of dithionite to an open cuvette containing oxidized, resting cytochrome oxidase. Relative dithionite concentration is monitored by the absorbance at 315 nm, while the enzyme's redox state is measured at 605 and 445 nm. The change in fluorescence is reported as the percent shift in the emission maximum with 20 nm taken as 100%. Upon addition of dithionite the fluorescence spectrum shifts immediately to 348 nm, whereas the enzyme obtains full reduction only after about 70 min. Dithionite depletion is essentially complete when reoxidation of the enzyme begins after 90 min. At this point the fluorescence emission spectrum has returned to 332 nm. Between 100 and 120 min, the sample is reoxidized as indicated by the return of the absorbance levels at 445 and 605 nm to the levels found with the resting enzyme. Results of a similar experiment, at higher enzyme concentration to monitor the redox level of Cu_A at 820 nm, demonstrate that under these conditions this center is fully reduced in the same manner as the heme centers (data not shown).

Carboxypeptidase A, a non-heme-containing enzyme, possesses intrinsic, steady-state tryptophan fluorescence that exhibits an emission maximum at 332 nm. Such an emission maximum value indicates that the tryptophan in this protein is in a hydrophobic environment and to that extent resembles the tryptophan found in cytochrome oxidase. Corrected and uncorrected fluorescence emission spectra of carboxypeptidase A in the presence and absence of dithionite are shown in Figure 3. In the absence of dithionite carboxypeptidase A has a fluorescence emission maximum at 332 nm, consistent with the value reported by Lakowicz and Weber (1973). In the presence of dithionite the uncorrected fluorescence emission maximum is red shifted to 345 nm. The corrected fluorescence emission spectrum of the dithionite-treated sample of carboxypeptidase A has a maximum at 332 nm.

When cytochrome oxidase is put under an atmosphere of CO, it forms a partially reduced, CO-bound species (Greenwood et al., 1974) in which cytochrome *a* and Cu_A remain oxidized and cytochrome a_3 and Cu_B are reduced. During this process CO and oxygen are turned over, and the mixed-valence enzyme begins to form when the system is anaerobic. Starting with a sample of mixed-valence CO-bound oxidase offers the technical advantage of beginning a reduction experiment in strictly anaerobic conditions, allowing the use of much lower levels of reductant to fully reduce the oxidase. Absorbance spectra of mixed-valence, CO-bound and fully reduced, CO-bound cytochrome oxidase are shown in Figure 4A. The Soret maximum of mixed-valence oxidase is at 430 nm, and upon addition of dithionite a shoulder develops at 445 nm, representing the reduction of cytochrome *a*. Mixed-valence, CO-bound cytochrome oxidase has an uncorrected, fluorescence emission spectrum that is identical with that of the oxidized, resting enzyme (Copeland et al., 1987). Reduction of mixed-valence, CO-bound enzyme with a slight excess of dithionite gives rise to an uncorrected fluorescence emission spectrum slightly red shifted to 332 nm (Figure 4B). When this spectrum is corrected for the absorbance due to dithionite, the fluorescence emission maximum is at 326 nm. This is 2 nm blue shifted in comparison to the fluorescence emission maxima of both resting and mixed-valence CO-bound cytochrome oxidase. Further addition of excess sodium dithionite to this sample does not change the visible absorbance spectrum, indicating that full reduction is achieved in the initial dithionite addition. Moreover, we have obtained the same result with

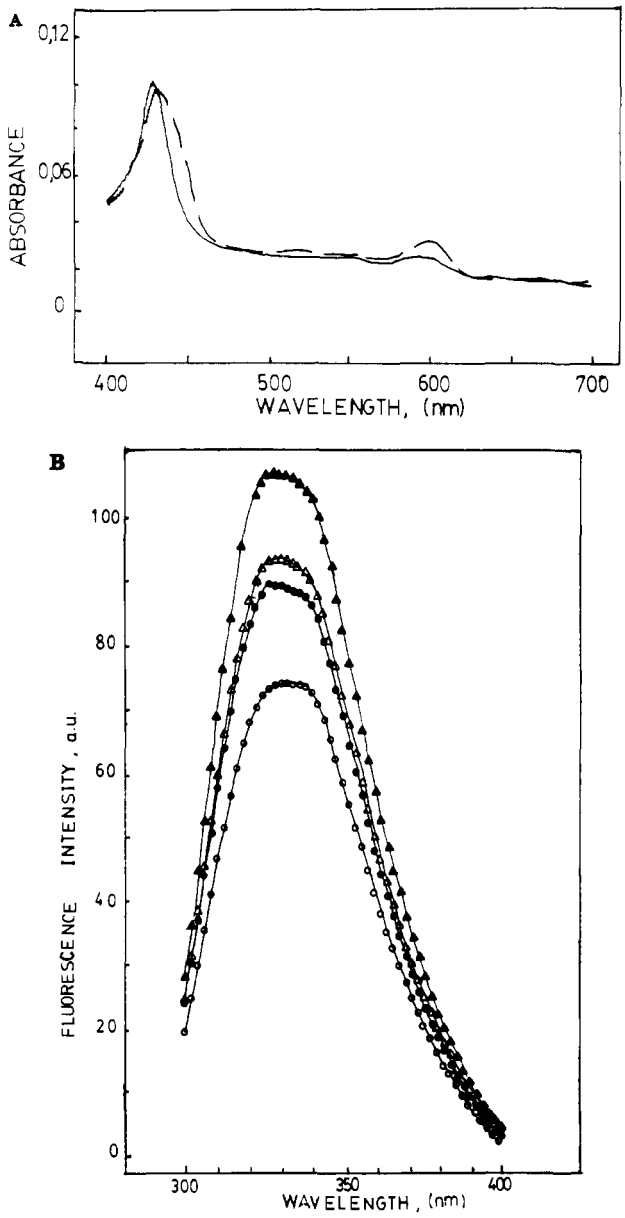

FIGURE 4: Absorbance and fluorescence spectra of dithionite-reduced, CO-bound cytochrome oxidase. (A) Reduction of mixed-valence, CO-bound cytochrome oxidase (0.5 μM) with sodium dithionite (50 μM): (—) mixed-valence CO-bound aa_3 and (--) fully reduced CO-bound aa_3. (B) Corrected (solid) and uncorrected (open) fluorescence emission spectra of mixed-valence CO-bound (▲, △) and fully reduced CO-bound (●, ○) cytochrome oxidase.

a sample of cytochrome oxidase prepared by the method of Hartzell and Beinert (1974). The observation indicates that the difference between our results and those of Copeland et al. (1987) are not due to a difference in the method of enzyme purification.

The application of eq 1, for the correction of fluorescence emission spectra for inner-filter effects, is problematic when absorbance values far exceed 0.20 (Lakowicz, 1983). This may account for the large differences in the emission intensities seen in the results of Figure 1. For this reason we have also used deazaflavin and EDTA to photoreduce mixed-valence CO-bound oxidase. Irradiation of mixed-valence, CO-bound oxidase for 1 min produces fully reduced enzyme (data not shown). Subsequent addition of excess dithionite does not cause any further change in absorbance. Unlike dithionite, deazaflavin does not absorb appreciably in the 320-nm region of the absorption spectrum at the concentrations required to reduce the enzyme. Hence, corrections for inner-filter effects are greatly decreased. Uncorrected fluorescence emission spectra for resting and photoreduced oxidase are essentially the same with fluorescence emission maxima at 328 nm. The corrected fluorescence emission spectrum of photoreduced enzyme is blue shifted by 4 nm to 324 nm.

We have also used a split fluorescence cell in which we place the oxidase and a solution of dithionite in separate compartments. In such an experiment the presence of dithionite causes the observed fluorescence emission spectrum to shift to 340 nm. When this spectrum is corrected for the inner-filter effect, due to dithionite absorbance, it is identical with that obtained with the resting enzyme.

Discussion

The recognition that cytochrome oxidase pumps protons has led to an intense effort to identify the particular redox center or centers that might serve a linking function between the free energy available from electron transfer and that required to move a proton across the barrier formed by the lipid membrane. Some workers (Artzatbanov et al., 1978; Callahan & Babcock, 1983) have argued that particular properties of cytochrome *a* make it the likely candidate, whereas Gelles et al. (1987) have presented a detailed model involving Cu_A directly in charge translocation and still others have suggested a role for the cytochrome a_3–Cu_B binuclear center (Chance, 1981; Mitchell, 1987; Baum et al., 1987). All of these models share the feature that a metal center plays a direct role in charge transfer by binding the translocated species in different states that are linked to redox turnover. These models propose a direct proton–electron coupling mechanism and do not require large-scale protein conformational changes.

A different class of model to those outlined above specifies conformational changes as intermediate states in proton transfer. Transitions between these conformers are driven by redox turnover at a particular metal center. Malmström and his coworkers have supplied evidence that reduction of both Cu_A and cytochrome *a* triggers a conformational change in the oxidase that results in an increased rate of cyanide binding at the cytochrome a_3–Cu_B center (Jensen et al., 1984; Scholes & Malmström, 1986) and increased rates of intramolecular electron transfer within the oxidase molecule (Fabien et al., 1987). They have proposed a model in which reduction of cytochrome *a* and Cu_A is linked, via a conformational change, to a protonation and deprotonation cycle that results in transmembrane proton movement (Thörnström et al., 1988).

Krab and Wikström (1987) have recently pointed out that neither the direct, ligand conduction nor the indirect, protein conformational models are exclusive. In order for protons to be translocated through cytochrome oxidase, some protein conformational rearrangement is required. The question remains whether or not this protein conformational change is large in scale and whether any of the metal centers play a direct role in binding the translocated ion. Tryptophan fluorescence should be a useful tool in discerning the difference between these two extreme possibilities. Intrinsic tryptophan fluorescence from cytochrome oxidase arises from tryptophan residues of the complex that are in a hydrophobic, protein environment located at about 30 Å from the strongly quenching heme centers; thus, they serve as a good probe of the global conformation of the oxidase complex (Hill et al., 1986). It has recently been reported that reduction of the oxidase causes a shift in the maximum of the tryptophan emission spectrum by 18 nm to the red (Copeland et al., 1987). This shift has been interpreted as being caused by a redox-linked conformational change in the oxidase molecule that is specifically caused by the reduction of Cu_A (Copeland et al.,

1987, 1988). These data give strong support to the idea that large-scale conformational changes in cytochrome oxidase may be important in the process of energy transduction (Thörnström et al., 1988; Nilsson et al., 1988). In this paper we show that reduction of cytochrome oxidase does not give rise to the shift in fluorescence reported by Copeland et al. (1987) and that this is not due to a difference in our preparative method. We suggest that the apparent spectral effect is due to the absorbance of the reductants in the region of the tryptophan emission spectrum. Correction of the dithionite-reduced spectrum or reduction of the oxidase by the photoreductant deazaflavin yields a tryptophan emission spectrum that is slightly blue shifted, by at most 4 nm, relative to that of the resting, oxidized enzyme. Our results argue against the view that large-scale, global conformational changes occur during the redox cycle of cytochrome oxidase. It is well recognized that such large-scale changes are not required in the functioning of a redox-linked proton pump (Gelles et al., 1987; Krab & Wikström, 1987). However, other approaches, such as subunit-specific antibody binding, imply that the oxidase's conformation is rearranged during its redox cycle (Freedman et al., 1988). It could be that the small shift we observe in the steady-state tryptophan fluorescence may be due to such changes. We are exploring these possibilities by measuring tryptophan lifetimes of the oxidase in different redox states. The participation of individual metal centers and more localized conformational changes in the proton translocation activity of cytochrome oxidase is still open to question.

Registry No. Cytochrome oxidase, 9001-16-5; tryptophan, 73-22-3; dithionite, 14844-07-6.

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