A Reexamination of Tryptophan Fluorescence in Cytochrome *c* Oxidase

Thesis by

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In Partial Fulfillment of the Requirements

for the Degree of

Master of Science

California Institute of Technology

Pasadena, California

1991

(Submitted May 31, 1991)

Acknowledgement

Several people have helped this work in crucial ways. I would like to thank Dr. Lutfur R. Khundkar and Dr. Joseph W. Perry for collaboration in measuring the tryptophan fluorescence lifetimes, Prof. Michael Dunn and Peter Brzovic for use of their stopped-flow fluorometer and rapid scanning stopped-flow spectrometer, and Prof. Bruce Hill and Dr. Dennis Rousseau for keeping us informed of findings in their laboratories.

Dr. Robert Copeland started this project and provided much personal encouragement. I am grateful to Prof. Sunney I. Chan for continually expecting precise scientific thinking and caring about my development.

This material is based upon work supported under a National Science Foundation Graduate Fellowship.

Finally I'd like to thank all the people who listened to my problems and helped me stay sane during my time at Caltech, especially Donna Smith, my roommates, Siang-Yang Tan, Norm & Nancy Rasmussen, The Caltech Graduate Bible Study, The Pecos Community, and the Cambria Community and Kinship. This thesis is dedicated to my friends in the Cambria Apartments.

"Unless the Lord builds the house,
those who build it labor in vain."

Psalm 127

Abstract

Cytochrome *c* oxidase links the reduction of oxygen with the pumping of protons across the inner mitochondrial membrane. Recently it has been shown that only two of the four electron transfers to the oxygen binding site are coupled with proton translocation. This discovery implies that there is a conformational switch between pumping and non-pumping forms of the enzyme. The expected properties of this unloaded/loaded conformational transition are discussed here and contrasted with gating conformational changes. Gating conformational transitions occur in each cycle of the pump when electrons and protons are alternatively accessible to either the inside or outside of the membrane. Such transitions are linked to the redox state of the coupling site. The unloaded/loaded transition, on the other hand, depends on the intermediate at the oxygen binding site and is not part of the pumping cycle.

A 16 nm red-shift in the tryptophan emission maximum of cytochrome oxidase upon reduction of the enzyme with dithionite was reported earlier [Copeland, R.A., Smith, P.A., & Chan, S.I. (1987) *Biochemistry 26*, 7311]. As such a redox–linked change could be an important probe of pumping transitions, the fluorescence change of cytochrome oxidase upon reduction with ascorbate and cytochrome *c* was measured. The absence of a shift led to the reevaluation of the earlier experiments for inner filter effects. The earlier experiments were reproduced with proper optical controls, and a method for reconstructing absorbance artifacts from the reported fluorescence spectra was devised. Tryptophan fluorescence lifetimes were also measured. All the data support the conclusion that the earlier reports of a redox-linked fluorescence change were artifacts and that the other transient changes in fluorescence which have been measured cannot be associated with either the unloaded/loaded transition or a gating transition.

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Part 1

Introduction

Cytochrome *c* oxidase spans the inner mitochondrial membrane and catalyzes the transfer of electrons from cytochrome *c* to molecular oxygen in the reaction:

$$4\ \text{Fe(cytochrome } c\text{)}^{2+} + O_2 + 4\ H^+ \rightarrow 4\ \text{Fe(cytochrome } c\text{)}^{3+} + 2\ H_2O.$$

As is shown in figure 1, the protons for this reaction come exclusively from inside the mitochondria (the mitochondrial matrix), while the electrons come from the cytoplasmic side of the membrane (the cytosol). The energy released by the reduction of a molecule of oxygen is used to pump up to four additional protons across the membrane (Thelen *et al.*, 1985; Wikström, 1977). During steady-state respiration, the proton concentration inside the mitochondria is one to two pH units higher than the concentration outside, and there is an electrostatic potential of about 180 mV across the membrane (Gelles *et al.*, 1986), giving a total protonmotive force of approximately 240 mV. Although the net driving force to pump protons against this gradient (the vectorial reaction) and selectively consume protons from the inside of the mitochondria (the scalar reaction) is 575 mV at standard conditions (298K, pH 7), not all of the four electron transfer steps are equally exothermic. It has recently been discovered that all the protons are pumped in just two of the electron transfer steps (Wikström, 1989). The mechanism by which the transfer of electrons to oxygen is coupled to the pumping of protons is a topic of current research. Efforts are under way in several laboratories to detect the conformational changes which must be associated with proton pumping. This thesis first describes the types of conformational changes that should be associated with proton pumping, then it details experiments evaluating the use of tryptophan fluorescence spectroscopy as a conformational probe.

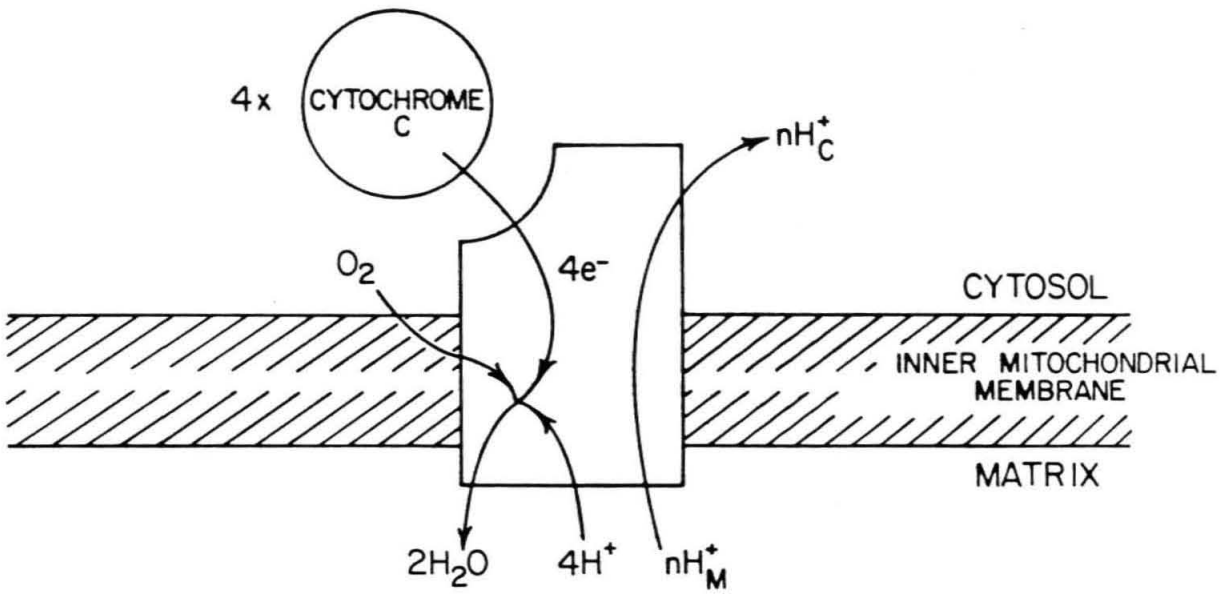


FIGURE 1. Schematic view of the major physiological functions of cytochrome *c* oxidase.

Cytochrome oxidase is a large protein consisting of three to thirteen subunits and having a molecular weight of up to 260,000 (for a review, see Wikström *et al.*, 1981). The three largest subunits are extremely well conserved in species as diverse as bacteria, yeast, and man. These three subunits, which constitute the entire bacterial protein, are genetically coded inside the mitochondria of eucaryotes, while the other more variable subunits are coded and synthesized in the cytoplasm. Subunits I and II are known to bind the redox-active metal centers of cytochrome oxidase, while subunit III can be removed by several methods without destroying the electron transfer activity of the enzyme (for reviews see Brunori *et al.*, 1987 and Prochaska & Fink, 1987). The removal of subunit III does, however, appear to halve the proton pumping stoichiometry. The labeling of subunit III with dicyclohexylcarbodiimide (DCCD)[1] also lowers the proton pumping ration to two protons per oxygen molecule. Furthermore, cytochrome oxidase from the bacteria *Paracoccus denitrificans*, from which subunit III is lost during purification, shows a halved proton pumping efficiency (Solioz *et al.*, 1982). It is unclear whether the removal of subunit III affects proton pumping directly, in that one of two pump sites is located in the subunit, or indirectly, either because subunit III has a regulatory function or because its removal destabilizes a pumping site in another part of the protein. Subunit III is an integral membrane protein, composed largely of hydrophobic residues forming putative transmembrane helices, and its removal tends to destabilize interaction between monomers that causes native cytochrome oxidase to be dimeric.

Electron transfer in cytochrome oxidase is mediated through four metal centers. Fe_a and Cu_A are located on the cytosolic side of the membrane and serve as the primary electron acceptors from cytochrome *c*. The standard reduction potentials of these centers is near that of cytochrome *c* ($E^{o\prime}$= 225 mV; Gelles *et al.*, 1986), and it has been widely assumed that one or both of these centers is the coupling site of electron transfer to proton pumping. The high potential metal centers, Fe_{a3} and Cu_B, are buried in the membrane

[1] Abbreviations used: cytochrome oxidase, ferrocytochrome *c* : oxygen oxidoreductase (EC 1.9.3.1) DCCD, dicyclohexylcarbodiimide; EPR, electron paramagnetic resonance; Hepes, N-2-(hydroxyethyl)piperazine-N´-2-ethanesulfonic acid; KDP, Potassium dihydrogen phosphate; Lauryl maltoside, dodecyl β-D-maltoside; pHMB, p-(hydroxymercuri)benzoate; Tris, tris(hydroxymethyl)-aminomethane; UV-vis, ultraviolet-visible.

and together form the binuclear oxygen binding site. In the enzyme as isolated (the "resting oxidase"), Fe_{a3} and Cu_B are strongly antiferromagnetically coupled through a bridging ligand. After one turnover, this ligand is displaced, yielding a much more active "pulsed oxidase." The distance between the high potential centers has been estimated at 0.5 nm in the nitric oxide inhibited enzyme (Chan *et al.*, 1982). Although both iron atoms are present as non-covalently bound heme A groups, only Fe_{a3} can bind exogenous ligands. The Cu_A site in cytochrome oxidase is spectroscopically unique. A broad optical absorption band at 830 nm and a distinctive EPR spectrum with unusually small and isotropic hyperfine splitting both occur when Cu_A is oxidized. These features have been attributed the coordination of copper to two sulfur ligands, as is shown in Figure 2 (Martin *et al.*, 1988). This site is subject to modification both by reaction with pHMB and by heating (Gelles & Chan, 1985; Li *et al.*, 1988). Because of its unique structure and the ease with which its ligands can be replaced, a redox-linked ligand rearrangement of Cu_A has been proposed as the means by which electron transfer and proton pumping are coupled in cytochrome oxidase (Gelles *et al.*, 1986).

The current understanding of the mechanism oxygen reduction in cytochrome oxidase is illustrated in figure 3 (Chan & Li, 1990). Oxygen binds to the cytochrome oxidase in which the binuclear center is reduced and rapidly receives two electrons, forming a peroxidic adduct, compound C. As more electrons are transferred from the low potential metal centers, first the O–O bond is broken, forming the ferryl compound, and finally oxygen is fully reduced, yielding the pulsed enzyme. The transfer of two more electrons from the low potential centers to the high potential centers (not shown in figure 3) completes the catalytic cycle and prepares the enzyme to bind oxygen again. The pumping of two protons is coupled to the reduction of compound C to the ferryl intermediate, and another two protons are pumped when the ferryl species is reduced to form the pulsed enzyme (Wikström, 1989). The electron transfers to the "unloaded" binuclear center are not coupled to proton transfer. When the catalytic cycle is divided in four parts, based on the four electron transfers from the low potential metal centers to the high potential centers, only one part is strongly exothermic under physiological conditions (Wikström, 1986,

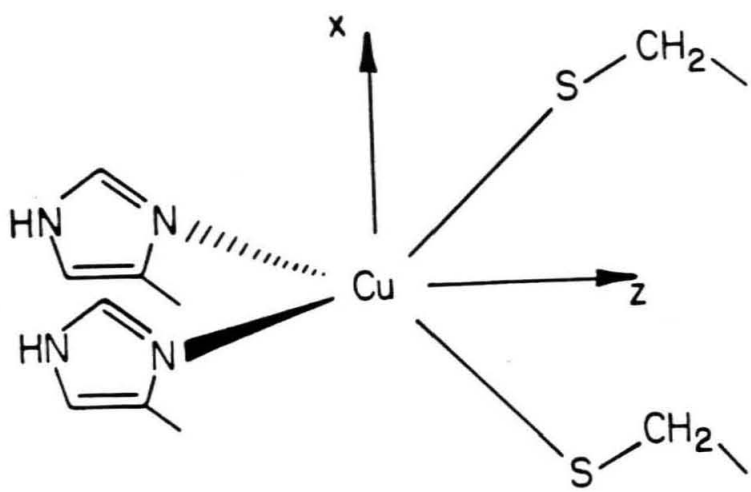


FIGURE 2. A model of the oxidized Cu_A site (from Martin *et al.*, 1988).

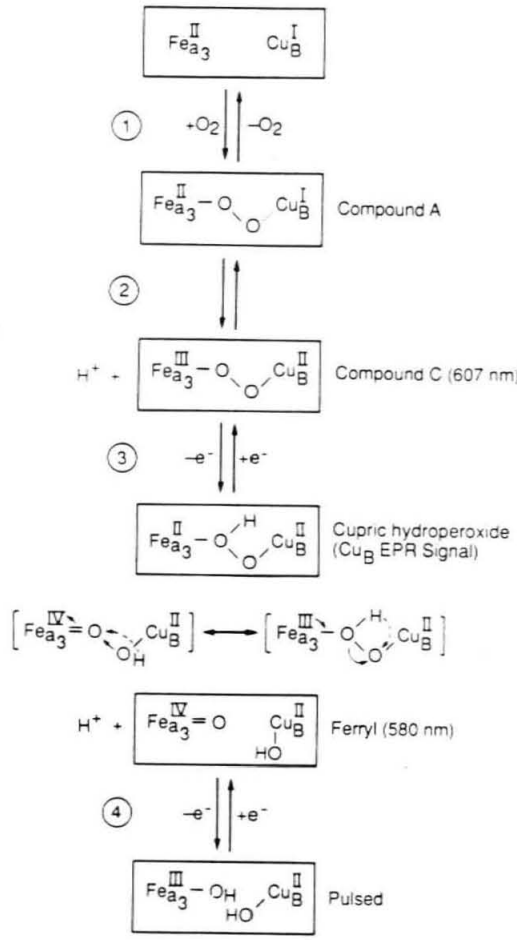


FIGURE 3. Scheme depicting the reduction of oxygen at the binuclear site of cytochrome oxidase. The four reduction steps of oxygen are numbered and the electron transfer steps from the low potential metal centers are also indicated. (From Chan and Li, 1990.)

1988). The irreversible part of the catalytic cycle includes the second unloaded electron transfer, the subsequent binding of oxygen to the protein, and the two electron reduction of oxygen to compound C. The existence of both coupled and uncoupled electron transfers from the low potential metal centers to the oxygen binding site requires a conformational switch between a pumping form of the enzyme and a non-pumping form. This may be accomplished either by changing the overall electron transfer route (for example by having loaded electron transfers from one of the low potential metal centers and unloaded transfers from the other center) or by disengaging and engaging a coupling mechanism along the same electron transfer route. The irreversible step of the catalytic cycle traps cytochrome oxidase in the loaded conformation. In itself, the unloaded to loaded conformational transition may be thermodynamically unfavorable, but it may be linked to a downhill process such as oxygen binding.

An analog of the T–R allosteric transition in hemoglobin upon binding of oxygen to the heme has been proposed for the unloaded/loaded transition in cytochrome oxidase (Larsen *et al.* 1991). This proposal highlights the fact that the conformational switch need not be linked to an electron transfer or to the redox state of any one metal center. It has been speculated that the unloaded/loaded transition may be the rate limiting step in enzyme turnover (Li, 1990), as the measured electron transfer rates are considerably faster than turnover. The R–T hemoglobin transition has been found by UV resonance Raman spectroscopy to occur in 20 μs (Spiro *et al.*, 1990), almost three orders of magnitude faster than cytochrome oxidase turnover. Even if the reverse T–R transition is considerably slower than the R–T transition, it is clear that such an allosteric conformational change is kinetically feasible for cytochrome oxidase. Indeed, it need not be the rate limiting step of catalysis. Whether or not the unloaded/loaded transition is linked to the binding of oxygen at Fe_{a3} , movements of the protein chain during an allosteric conformational change are likely to be detectable by UV resonance Raman spectroscopy because of this technique's sensitivity to the environment of individual residues. Another factor which could play a role in driving the unloaded/loaded transition is the electrostatic repulsion between the reduced metal centers of the oxygen binding site. The repulsion between two unit charges

which are separated by 0.5 nm of water (with a dielectric constant of 80) is about 35 mV. In the reduced form of the enzyme, there may be as many as five charges at the binuclear center, even after the peroxidic adduct has formed. Thus the electronic repulsions become a considerable energetic factor.

In addition to conformational transitions which switch cytochrome oxidase between pumping and non-pumping states, a pumping mechanism requires a series of gating conformational changes. Separate input and output states of the transducing site are required both for electrons (electron gating) and for protons (proton gating), and these states must be linked so that an electron cannot pass though the pump site without the corresponding one or two protons being translocated (Blair *et al.*, 1986). Gating conformational transitions should be linked to the redox state of the transduction site.

In order for a proton pump to achieve maximum power output, some leakage is necessary (Blair, *et al.*, 1986). When the membrane becomes fully charged, uncoupling reactions allow electron transfer rates to remain fast in exchange for a loss in pumping efficiency. Several types of leakage can be envisioned. First, there can be pump slippage, where the electron or proton gating is circumvented so that an electron is transferred without a proton being translocated. However leakage might also occur at the loaded/unloaded switching level, especially if the allosteric transition were sensitive to membrane potential. A number of transmembrane ionic channels are conformationally sensitive to membrane potential. At high membrane potentials, the reduction of the ferryl intermediate to the pulsed enzyme is energetically uphill when coupled to the pumping of two protons. The selective uncoupling of proton pumping at this step would be the most effective means of increasing electron transfer rate at the expense of efficiency. A third, similar type of leakage, would be if subunit III had a regulatory function, coupling proton pumping under low membrane potential conditions, and uncoupling the pumping under high potential conditions. Structural similarities between subunit III and voltage or ion sensing membrane channels should be looked for. A very illuminating but technically difficult experiment would be to co-reconsitute subunit III depleted cytochrome oxidase with ATPase and to test the dependence of the ferryl/pulsed equilibrium on membrane

potential. This experiment, analogous to that performed by Wikström (1989), would show whether the 50 % pumping efficiency of subunit III depleted enzyme is due to a 50 % efficiency at each of the two pumping steps or to complete abolition of one step and retention of the other step.

Part 2

A Reexamination of Tryptophan Fluorescence in Cytochrome *c* Oxidase

Abstract

When cytochrome *c* oxidase was reduced with ascorbate and cytochrome *c*, the tryptophan fluorescence emission spectrum was found to be the same as for the oxidized enzyme. It was reported earlier [Copeland, R.A., Smith, P.A., and Chan, S.I. (1987) *Biochemistry 26*, 7311] that reduction of cytochrome *c* oxidase causes a 16 nm red-shift in the fluorescence emission spectrum. Reproduction of the earlier experiments suggested that the apparent shift in fluorescence might be an inner filter effect of dithionite absorption. A method for reconstructing the absorption difference spectrum from two fluorescence spectra differing only by inner filter effects was used to verify that dithionite could account for the observed fluorescence shift. The stopped-flow mixing of cytochrome oxidase and cytochrome *c*, which was reported to result in a rapid increase in fluorescence, was observed by rapid-scanning absorption spectroscopy. Absorption changes caused by the oxidation of cytochrome *c* account for the rapid fluorescence changes. However, it was observed that some small fluorescence changes do occur when cytochrome oxidase is diluted in buffer, perhaps due to protein denaturation. No quenching of the cytochrome oxidase tryptophan fluorescence by cesium was measured in either the oxidized or reduced enzyme. Tryptophan fluorescence lifetimes were measured by time-correlated single photon counting. The fluorescence decay is multiexponential and depends on the emission wavelength rather than on the reduction state of the enzyme. A triexponential fit of the decay at 320 nm yields lifetimes of 3.5 ns, 0.9 ns, and 0.13 ns .

Large-scale protein conformational changes have been effectively studied by fluorescence spectroscopy. The predominant fluorophore in proteins, tryptophan, is very sensitive to changes in solvation (Lakowicz, 1983). The intrinsic fluorescence of cytochrome oxidase is dominated by tryptophan residues buried in the interior of the enzyme and distant from the heme groups (Hill *et al.*, 1986). Thus tryptophan fluorescence provides information about the oxidase structure away from the sites of other intrinsic or extrinsic probes. Copeland *et al.* (1987) reported that the fluorescence maximum of cytochrome oxidase shifts from 329 nm to 345 nm upon reduction of the enzyme, suggesting that the protein undergoes a conformational change which causes some tryptophan residues to be more solvent exposed.

A major complication in fluorescence studies is the apparent change in fluorescence due to changes in the absorbance of fluorophores or other species in the reaction mixture. In an attempt to minimize such inner filter effects, cytochrome oxidase was reduced by different means (ascorbate with a catalytic amount of cytochrome *c*), and the fluorescence spectrum was recorded. As these results were inconsistent with Copeland *et al.* (1987), an effort was made to reproduce the earlier experiments. Meanwhile, other laboratories reported data inconsistent with a shift in tryptophan fluorescence (Ferreira–Rajabi & Hill, 1989, Dennis Rousseau, personal communication). In this paper we report new data showing that the tryptophan fluorescence of cytochrome oxidase does not change upon reduction of the enzyme and we explain the origins of the data reported by Copeland *et al.*

Materials and Methods

Materials. Sodium dithionite was purchased from GFS Chemicals. Potassium ascorbate was recrystallized and stored as a concentrated solution at – 80 ^{o}C until used. Argon for anaerobic work was scrubbed for oxygen by a column of manganese oxide on vermiculite.

Cytochrome *c* oxidase was isolated by the method of Harzell and Beinert (1974). The enzyme was solubilized in 0.05M Hepes buffer, pH 7.4, containing 0.5% Tween-20

(Sigma) and stored at –80 ^{o}C until used. Samples were diluted to the desired concentration in a 0.5% lauryl maltoside (Calbiochem), 50 mM Tris, pH 7.7, 50 mM NaCl buffer. The buffer was stored overnight at 2 ^{o}C and filtered to removed any aggregated detergent before addition of enzyme.

Equine ferricyctochrome *c* was purchased from Sigma (type VI). The reduced form of the enzyme was prepared by addition of excess sodium dithionite to a 1 – 2 mM solution of cytochrome *c*, and excess dithionite was removed by gel chromatography using two Sephadex G-25 centricolumns (Neal & Florini, 1973, Penefsky, 1977). Extinction coefficients reported by Margoliash & Frohwirt (1959) were used to assess the concentration and extent of reduction of the enzyme. For the reduced enzyme, an extinction coefficient of 27.7 $mM^{-1}cm^{-1}$ at 550 nm was used. For experiments involving the mixing of comparable volumes of cytochrome *c* and cytochrome oxidase (such as the stopped-flow samples), the cytochrome *c* was diluted to the desired concentration in the same lauryl maltoside buffer as the oxidase. For experiments involving a catalytic amount of cytochrome *c*, detergent was omitted from the buffer.

Sample Preparation. The cyanide derivative of cytochrome oxidase was prepared by incubation of the stock enzyme with 20 mM KCN for 24 to 72 hours at 4 ^{o}C, followed by centrifugation at 29,000 g to remove denatured protein. The enzyme was then diluted to 2.3 μM in lauryl maltoside buffer containing 4 mM KCN.

Reduced cytochrome oxidase was prepared by addition of either dithionite or potassium ascorbate and cytochrome *c*. As described earlier (Copeland *et al.*, 1987), 30 – 100 μL of a freshly prepared dithionite solution was added to approximately 3.5 ml of 1.8 μM oxidase which had been flushed with nitrogen for 10 minutes. After approximately five minutes spectra were recorded. Optical spectra were taken before and after the fluorescence spectra in order to assess the decay of dithionite during the scan. Ascorbate/cytochrome *c* reduction was accomplished by placing 1.6 – 1.8 μM cytochrome oxidase in a fluorescence

cell containing the reductants in a sidearm. After evacuating the cell and flushing with 1 atm. argon three times, an optical spectrum was recorded and the reactants were mixed, resulting in 0.01 mM ascorbate and 0.4 – 0.8 μM cytochrome *c*. After incubation overnight, the extent of reduction was assessed spectroscopically at the wavelength pairs 605 nm/ 622 nm and 444.5 nm/ 462 nm using the extinction coefficients of Blair *et al.* (1982). When the fluorescence spectra and lifetimes had been recorded, solid sodium dithionite was added to the sample under an argon atmosphere to record spectral values for full reduction. In some cases, prior to the addition of dithionite, the reoxidized enzyme was prepared by opening the sample to air for 10 minutes. The Soret and alpha band absorption increases in the ascorbate/cytochrome *c* reduced enzyme were 85 – 100% of the increases in the dithionite reduced enzyme.

Fluorescence quenching samples were prepared by the addition of solid CsCl to a final concentration of 0.5M.

Optical Absorption Spectroscopy. UV-vis absorption spectra were taken at 20 ^{o}C using a Beckman DU-7 HS scanning spectrophotometer. Base-line spectra were subtracted digitally.

Rapid-scanning stopped-flow spectra were obtained using a hybrid system consisting of the Durrum D-110 Kel F flow and illumination systems and the Princeton Applied Research OMA-2 multichannel analyzer, 1218 controller, and 1412 photodiode array detector as described in Sartorius, *et al.* (1987). The repetitive scan rate was 7.5 ms/scan. Difference spectra were calculated through subtraction of the last scan (at 1.5 sec).

Fluorescence Spectroscopy. Fluorescence spectra and time courses were recorded on a SLM 4800 spectrofluorometer equipped with a SMC–210 monochrometer controller and SE–480–485 electronics which was interfaced to an IBM XT computer. The beam from a xenon arc-lamp was split for reference against a solution of 3g/L rhodamine B in ethylene glycol. For most samples, a 1 cm x 1 cm quartz cuvette was used, but emission inner filter effects could be reduced by using a 1 cm x 4 mm cuvette, illuminated along the 1 cm path.

Stopped flow fluorescence spectra were obtained using a Durrum stopped-flow

apparatus with the photomultiplier tube mounted perpendicular to the excitation beam path as described elsewhere (Dunn *et al.*, 1979, 1980). Unless otherwise noted, a Rolyn 65.1010 filter (bandpass 300–400 nm) and a Rolyn 65.1620 filter (highpass 310 nm) were placed between the sample and the photomultiplier tube, producing a transmittance window from 310 nm to 390 nm. To assess the dependence of the signal on emission wavelength, the highpass filter was substituted by one or two Rolyn 65.1625 filters (highpass 335 nm) and both filters were replaced by a Corion special bandpass (313 nm ± 20 nm) filter. Photomultiplier voltages were recorded and fit to a single-exponential curve of the form $F = A + B\ \{1 - \exp(Ct)\}$, where F is the measured fluorescence, t is time, and A, B and C are the parameters of the fit. The change of fluorescence reported is 100% (B/A) and the time constant is C.

Fluorescence lifetimes were measured by time-correlated single photon counting. An excitation frequency of 290 nm was generated using a synchronous pumped dye laser and a frequency doubling KDP crystal, achieving a time resolution of 70 ps. Fluorescence emission was measured at 320 nm, 340 nm, and 370 nm with a wavelength resolution of 10 – 20 nm. The time-course was recorded using a multi-channel analyzer set to 12.766 ps per channel. Lifetimes were determined by fitting 800 channels, beginning with channel 65, to a triexponential curve.

Inner Filter Correction and Reconstruction. Fluorescence spectra were corrected for inner filter effects by using the expression (Lakowicz, 1983):

$$F_{corr} = F_{obs}\ \text{antilog}\{(OD_{ex} + OD_{em})/2\} \qquad (1)$$

where F_{corr} is the corrected fluorescence intensity, F_{obs} is the observed fluorescence intensity, and OD_{ex} and OD_{em} are the optical densities of the sample at the excitation and emission wavelengths respectively. This expression can also be used to generate the optical spectrum of a species causing an inner filter effect in an uncorrected fluorescence spectrum. If the uncorrected fluorescence spectra of two samples (x and y) differ only because of inner filter effects and instrument gain, then we have:

$$F_{obs}(x)\ \text{antilog}\{[OD_{ex}(x) + OD_{em}(x)]/2\}/\ g(x) = F_{corr}$$
$$= F_{obs}(y)\ \text{antilog}\{[OD_{ex}(y) + OD_{em}(y)]/2\}/g(y)$$

where g(x) and g(y) are the instrument gains of spectra x and y respectively. This can be rearranged to give:

$$\log [F_{obs}(x)/F_{obs}(y)] = \log [g(x)/g(y)] + [OD_{ex}(y) - OD_{ex}(x)]/2 + [OD_{em}(y) - OD_{em}(x)]/2 \quad (2)$$

If two fluorescence emission spectra are being compared, then the first and second terms on the right hand side of this equation are constants, while the third term is proportional to the optical difference spectrum between samples x and y. If fluorescence excitation spectra are being compared, then the first and third terms determine the baseline, while the second term produces the difference spectrum. The left hand side of this equation is calculated directly from the experimental spectra.

Results and Discussion

The study of tryptophan fluorescence in reduced or partially reduced forms of cytochrome *c* oxidase is hampered by the optical absorbances of most electron donors in the near ultraviolet spectrum. The absorption spectrum of dithionite, shown in Figure 4, is a broad peak centered at 315 nm, with an extinction coefficient of 6.9 $mM^{-1}cm^{-1}$ (Creutz & Sutin, 1974). Aerobic solutions containing dilute dithionite were found to be unstable due to the rapid reaction of dithionite with oxygen. Therefore accurate inner filter corrections to the fluorescence of dithionite-reduced cytochrome oxidase could be applied only if the optical and fluorescence spectra were taken without any intervening delay. Uncorrected fluorescence spectra of oxidized and dithionite-reduced cytochrome oxidase are shown along with the corresponding optical spectra in Figure 5. Although minimal dithionite was used, the fluorescence maximum undergoes an apparent red-shift of 2 nm. The corrected spectra show no shift.

The observed lack of a shift in the fluorescence spectrum of cytochrome oxidase upon reduction with dithionite is incompatible with the observations of Copeland *et al.* (1987). Therefore equation (2) was applied to the data reported in Figure 2 of Copeland *et al.* (1987) to generate the optical spectrum required to produce such a shift through inner filter

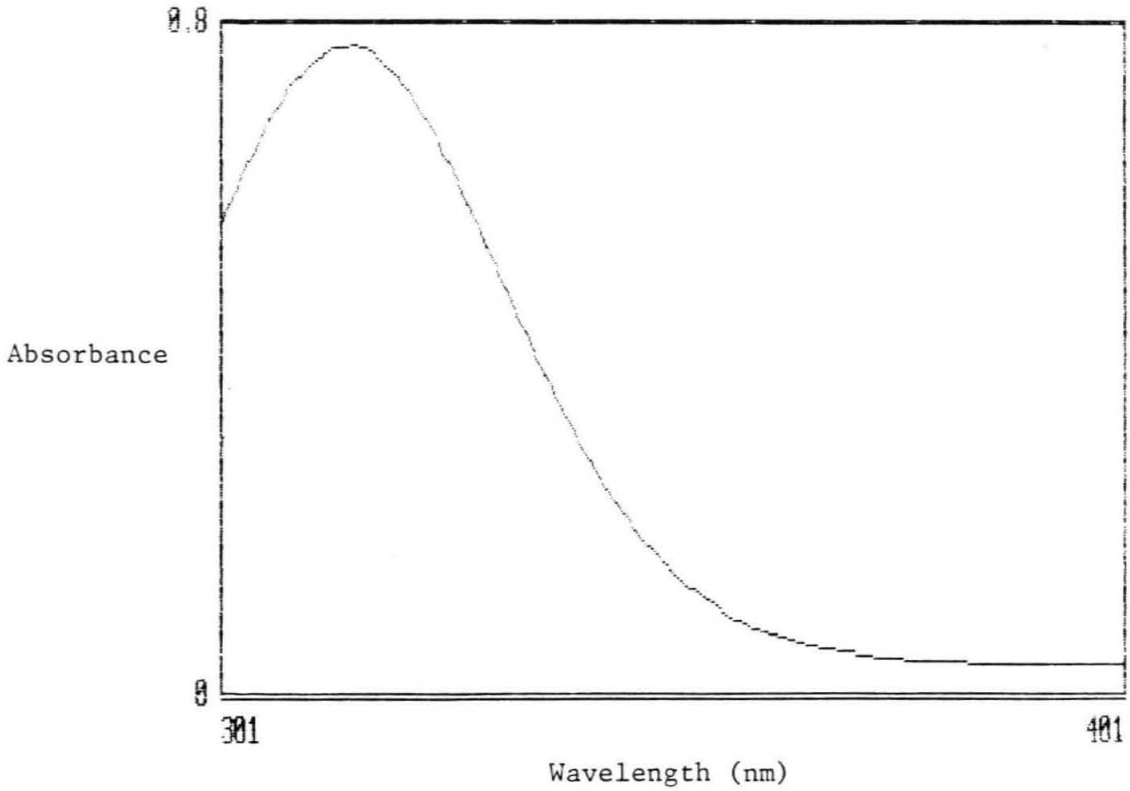


FIGURE 4. Absorbance spectrum of approximately 0.1 mM sodium dithionite.

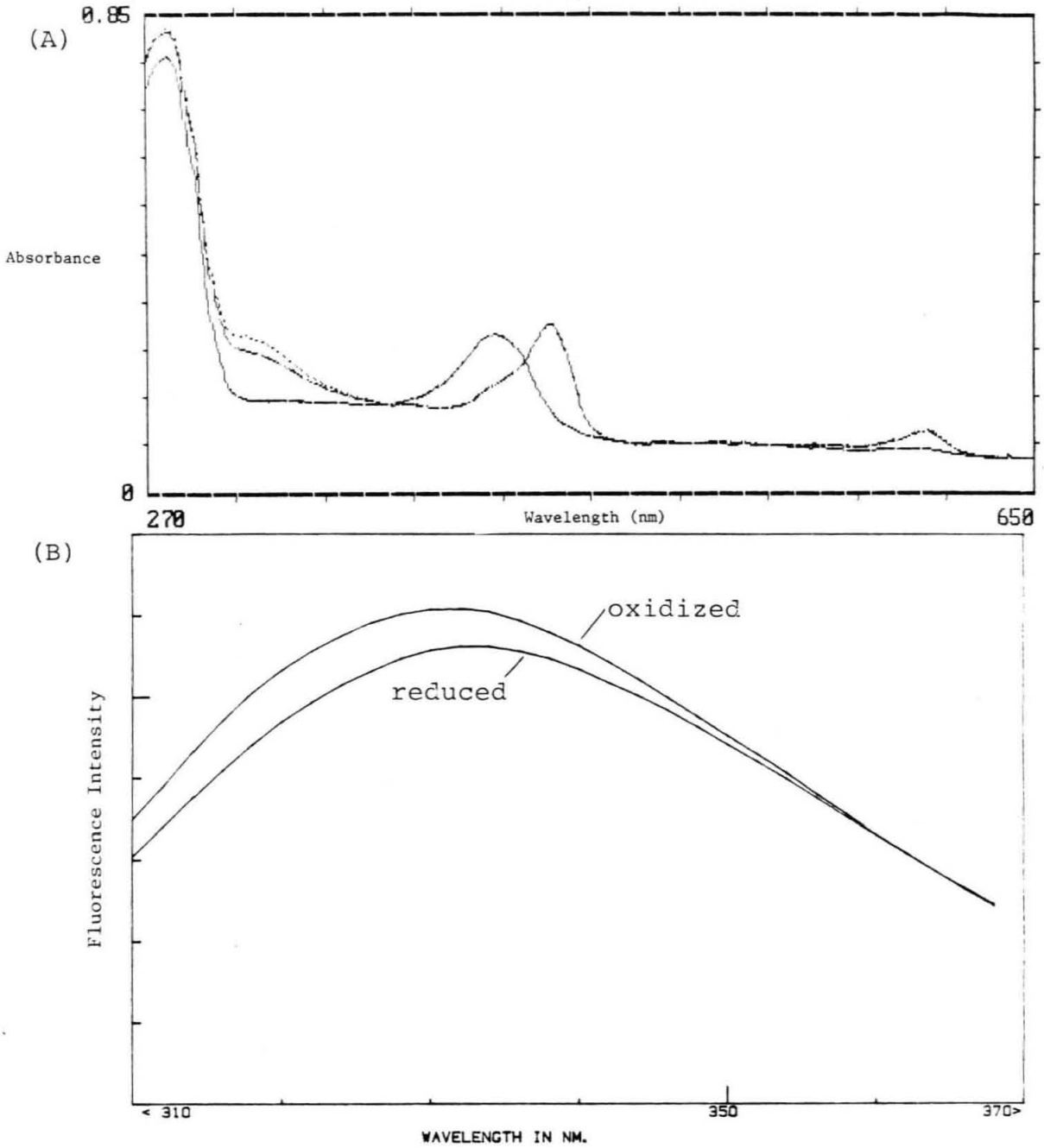


FIGURE 5. A. Absorption spectra of cytochrome oxidase in the resting oxidized form (solid line), after incubation with dithionite for 10 minutes (dotted line), and incubation with dithionite for 13 1/2 minutes (dashed line). B.Uncorrected fluorescence emission spectra of cytochrome oxidase in the resting oxidized form and after incubation with dithionite for 11 minutes. Excitation at 270 nm, excitation slit width 8 nm, emission slit width 4 nm.

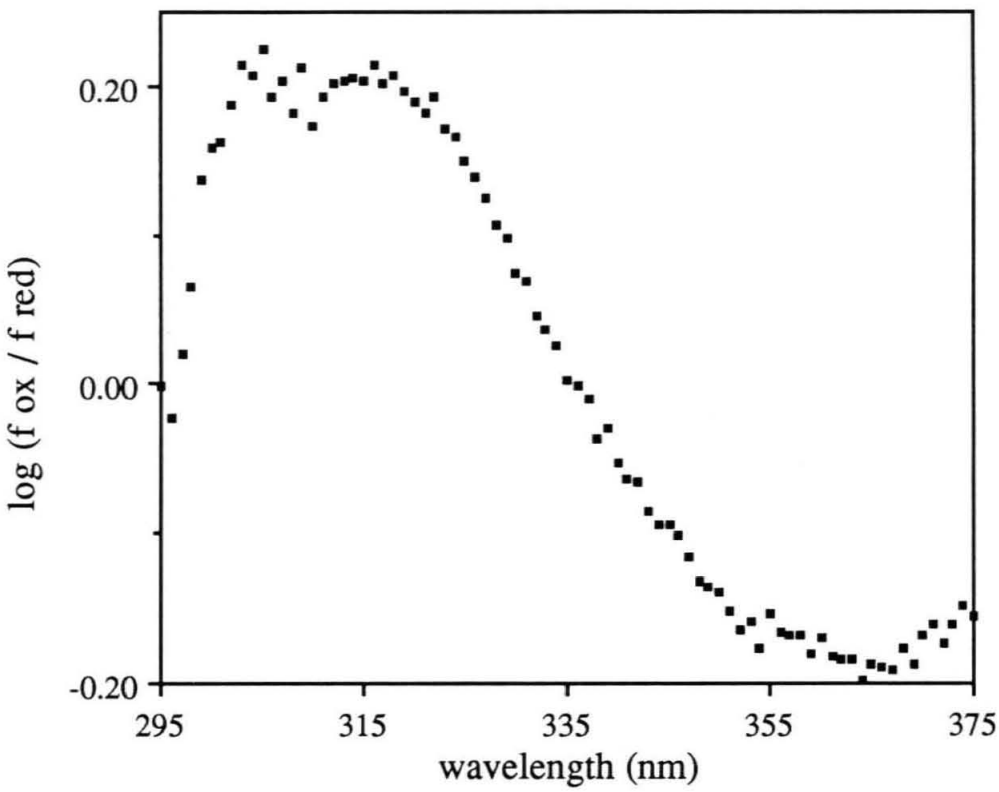


FIGURE 6. Reconstruction of the absorption difference spectrum between dithionite-reduced and reoxidized cytochrome oxidase from fluorescence data reported in Copeland *et al.* (1987). Details in text.

effects. The result of our back-calculation, shown in Figure 6, clearly indicates that the presence of approximately 0.12 mM dithionite would have been sufficient to cause the fluorescence red-shift in the uncorrected data. The actual corrections used reflect a concentration of only about 0.02 mM dithionite. The rapidly changing optical spectrum or an improperly mixed sample could have resulted inadequate inner filter corrections. We were also unable to reproduce any large increase in fluorescence upon reduction of cytochrome oxidase. The most plausible explanation for the reported increase is that the fluorometer gain was inadvertently adjusted between samples. This change in fluorometer gain manifests itself in the negative baseline of Figure 6. The combination of an apparent increase in fluorescence with a red-shift of the peak seemed to rule out inner filter effects in the original study.

The absorption spectrum of ascorbate, with a maximum at 265 nm and very little absorption above 300 nm, does not interfere with tryptophan fluorescence emission. However, the catalytic amounts of cytochrome *c* required to transfer electrons from ascorbate to cytochrome oxidase do cause a small inner filter effect. Figure 7 demonstrates that oxidized and reduced cytochrome oxidase show identical corrected fluorescence spectra.

The presence of 0.5 M CsCl did not affect the steady-state fluorescence of cytochrome oxidase in either the oxidized or reduced forms. The data used in constructing the Stern-Volmer plot in Copeland *et al.* (1987) clearly show not only decreases in fluorescence, but also the peak shifts associated with dithionite inner filter effects (Figure 8A). In the original study, the optical spectra associated with these fluorescence spectra were cursorily checked to verify that approximately the same amount of dithionite was used in each, and the spectra were not recorded. However, it is possible to reconstruct absorbance difference spectra using equation (2). The results, shown in Figure 8B, clearly implicate dithionite as the cause of the fluorescence decreases.

Further evidence that the tryptophan fluorescence of cytochrome oxidase is identical in the oxidized and reduced enzyme and that cesium does not quench either form was obtained through studying the fluorescence lifetimes of the protein. The fluorescence

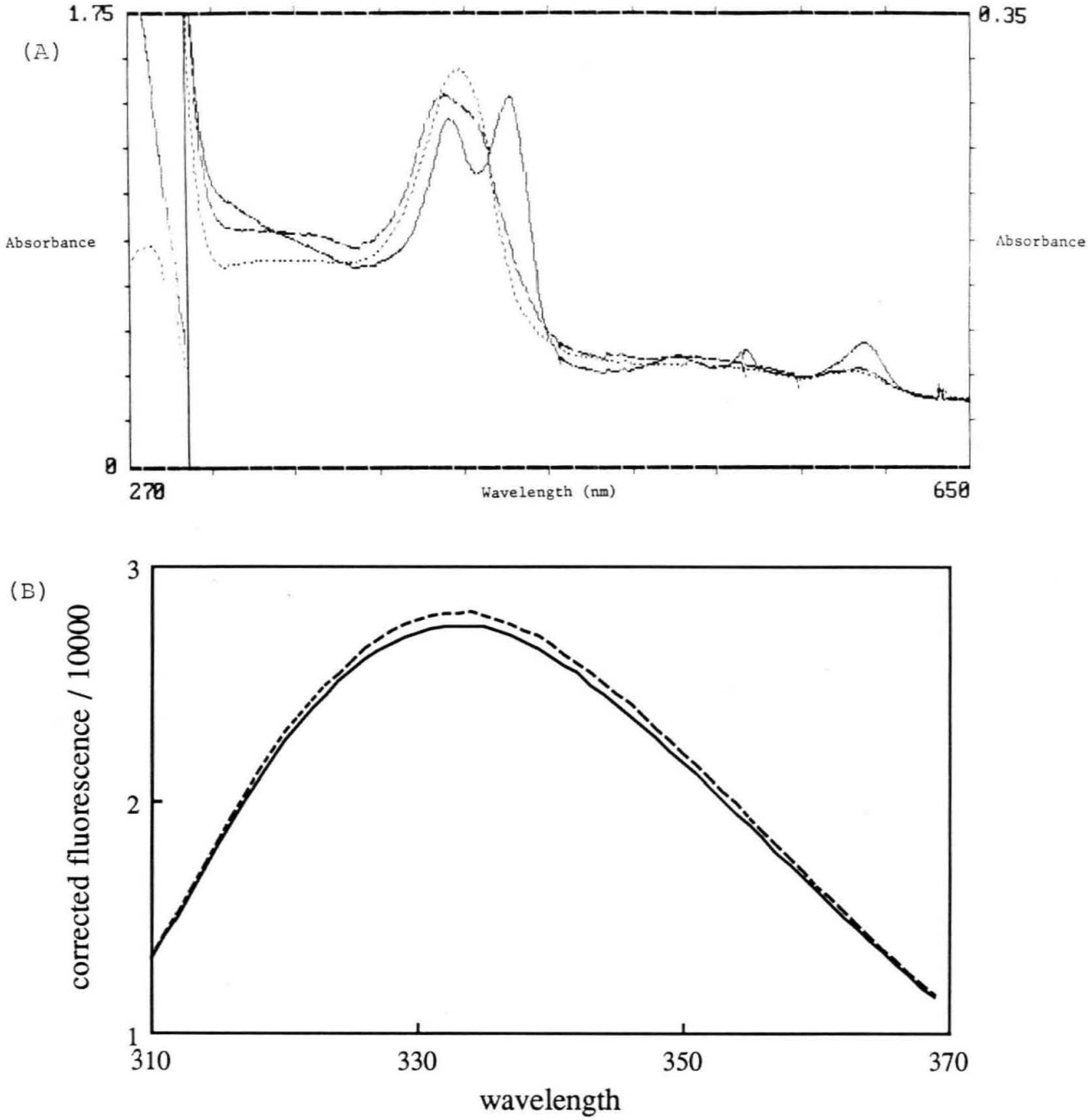


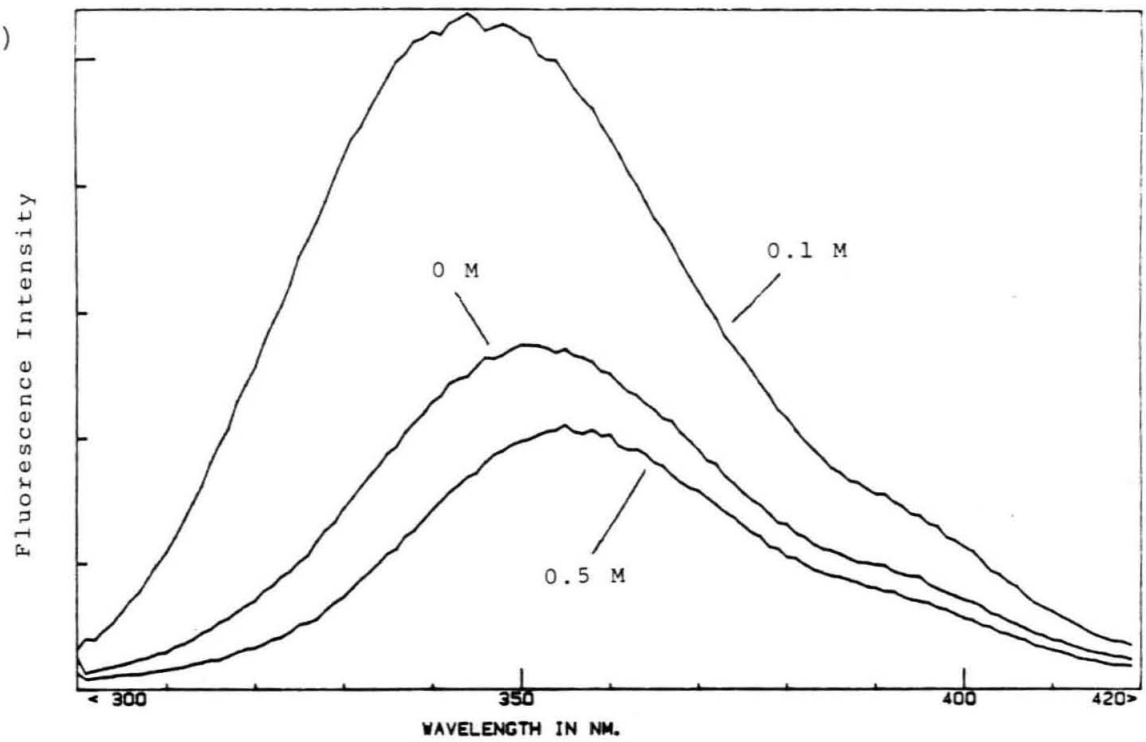
FIGURE 7. A. Absorption spectra of cytochrome oxidase in the resting form (dotted line), after incubation with 0.1 mM potassium ascorbate and 0.7 μM cytochrome *c* for 23 hours (solid line), and after reoxidation (dashed line). This sample contained 0.5M CsCl. B. Corrected fluorescence emission spectra of reduced oxidase (solid line) and reoxidized oxidase (dashed line). Excitation at 290 nm with a slit width of 8 nm. Emission slit width of 4 nm.

FIGURE 8.

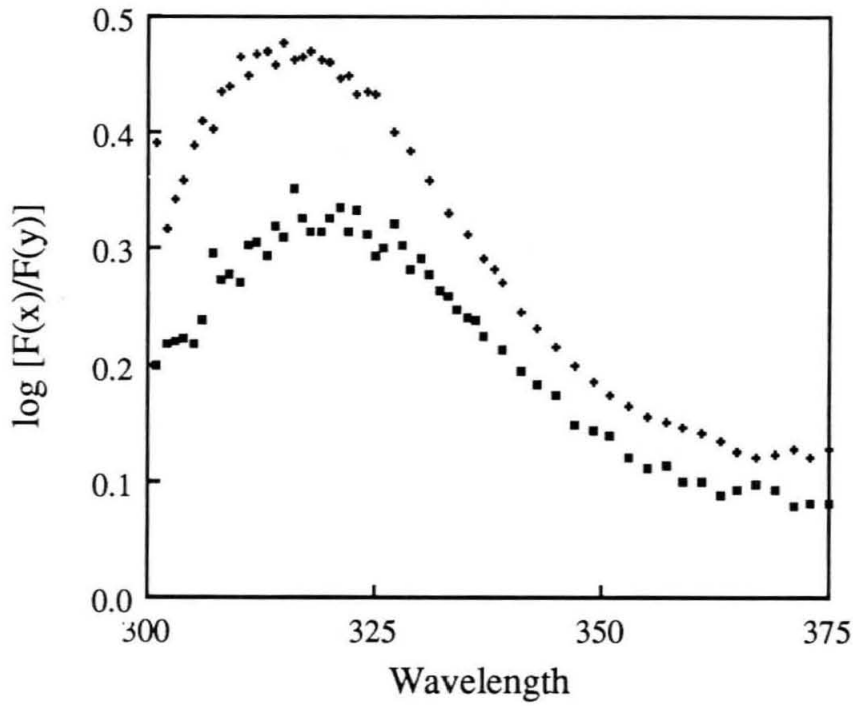
A. Uncorrected fluorescence spectra of dithionite reduced cytochrome oxidase containing 0 M, 0.1 M, and 0.5 M CsCl. Data from Copeland *et al.* (1987).

B. Reconstruction of absorption difference spectra from the fluorescence spectra in A. The abscissa indicates log {F[0M CsCl] / F[0.5M CsCl]} (squares) or log {F[0.1M CsCl] / F[0M CsCl]} – 0.1 (crosses). See text for further details.

(A)


Fluorescence Intensity
0 M
0.1 M
0.5 M
< 300
350
400
420>
WAVELENGTH IN NM.

(B)


log [F(x)/F(y)]
0.5
0.4
0.3
0.2
0.1
0.0
300
325
350
375
Wavelength

decays of oxidized and ascorbate/cytochrome *c* reduced enzyme, with and without 0.5 M CsCl, were essentially the identical. The fluorescence decay, shown in Figure 9, was multiexponential and depended on the emission wavelength. At higher emission wavelengths, the initial decay was faster but the long lifetime component decayed more slowly. The fluorescence lifetimes of individual tryptophan residues or homogenous groups of residues cannot be identified from the observed fluorescence decay. The decay represents a distribution of many lifetimes that can be characterized by a triexponential function. At an emission wavelength of 320 nm the decay is described by lifetimes of 3.5 ns (20%), 0.9 ns (40%), and 0.13 ns (40%), while at 370 nm the distribution is fit by lifetimes of 4.2 ns (34%), 1.1 ns (50%), and 0.24 ns (16%).

The corrected fluorescence spectra of oxidized and dithionite-reduced cyanide-inhibited cytochrome oxidase, shown in figure 10, are also identical. In no form of the enzyme did we observe a redox-linked fluorescence change.

The reaction of cytochrome oxidase with ferrocytochrome *c* was reported by Copeland *et al.* (1987) to be accompanied by a small increase in fluorescence. We have investigated the absorbance changes of this reaction with a rapid-scanning stopped-flow spectrophotometer. Difference spectra of the reaction, shown in Figure 11, are the same as the difference spectrum of reduced minus oxidized cytochrome *c*. The absorbance changes caused by the oxidation of cytochrome *c* occur at the same rate as the apparent fluorescence changes, suggesting that the fluorescence changes may be due to an inner filter effect.

As Table 1 indicates, the extent of the fluorescence rise depends only on the concentration of cytochrome *c*, not on the concentration of cytochrome oxidase. The time course of the fluorescence rise, on the other hand, depends on the concentration of cytochrome oxidase. As is shown in Table 2, a decrease of cytochrome oxidase in the reaction mixture by more than two orders of magnitude slows the reaction from less than a second to more than a minute without changing the magnitude of the fluorescence increase. Cytochrome oxidase is necessary for the fluorescence increase only insofar as it catalyzes

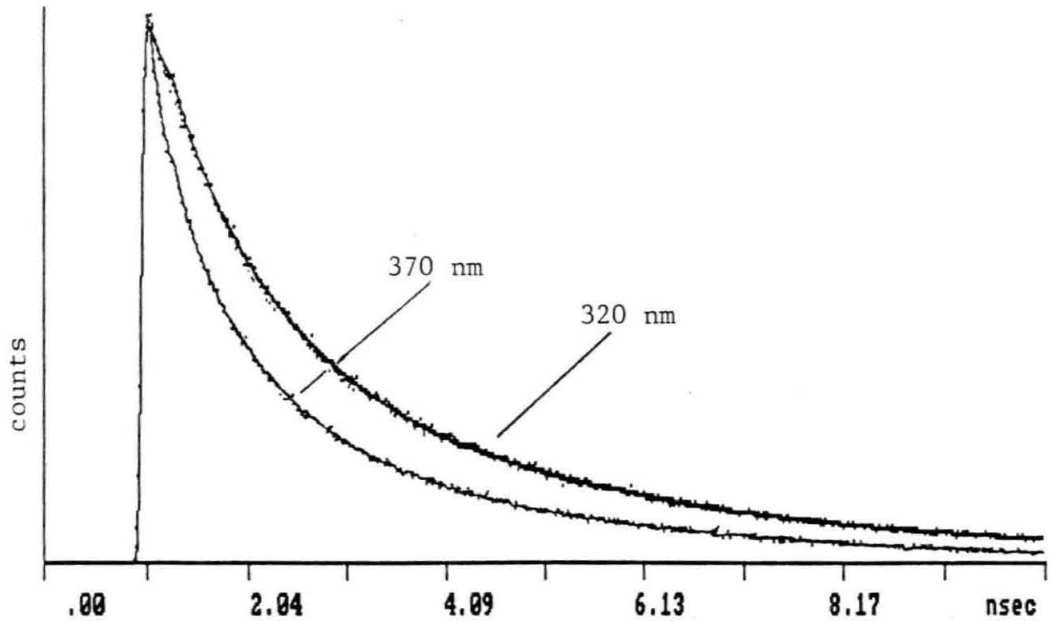


FIGURE 9. Fluorescence decays of acsorbate/cytochrome *c* reduced cytochrome oxidase at 320 nm and 370 nm. The data, indicated by dots, are fit by triexponential decay curves.

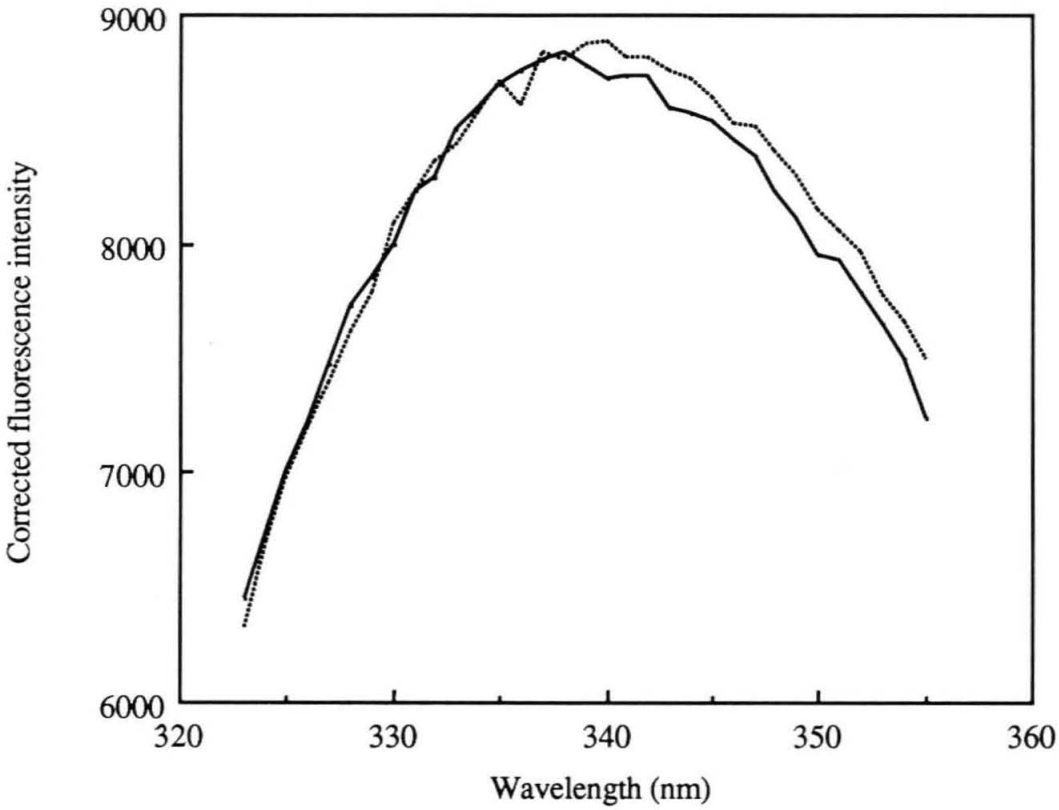
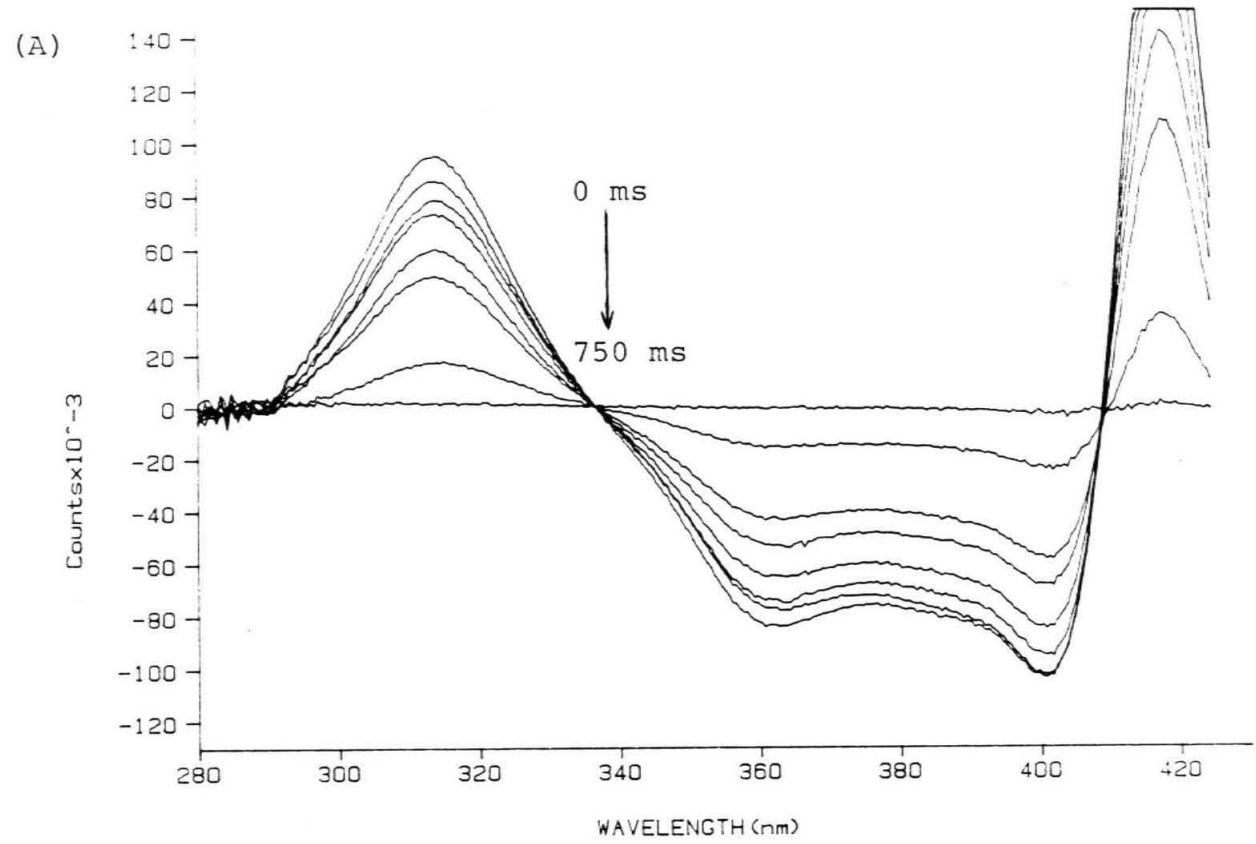


FIGURE 10. Corrected fluorescence spectra of cyanide-inhibited cytochrome oxidase in the oxidized (solid line) and dithionite-reduced forms (dotted line). Spectra were measured in a 10mm x 4mm cell with excitation at 290 nm, excitation and emission slit widths of 8.

FIGURE 11.

Rapid-scanning stopped-flow difference absorption spectra of the mixing of 12.4 μM cytochrome *c* with 1.55 μM cytochrome oxidase. The spectrum at 1.5 s was subtracted from spectra at (A) 0 ms, 7.5 ms, 22.5 ms, 45 ms, 75 ms, 105 ms, 240 ms, and 750 ms, (B) 7.5 ms, 60 ms, 90 ms, 150 ms, 330 ms, and 750 ms.


(A)
0 ms
750 ms
Countsx10^-3
WAVELENGTH (nm)

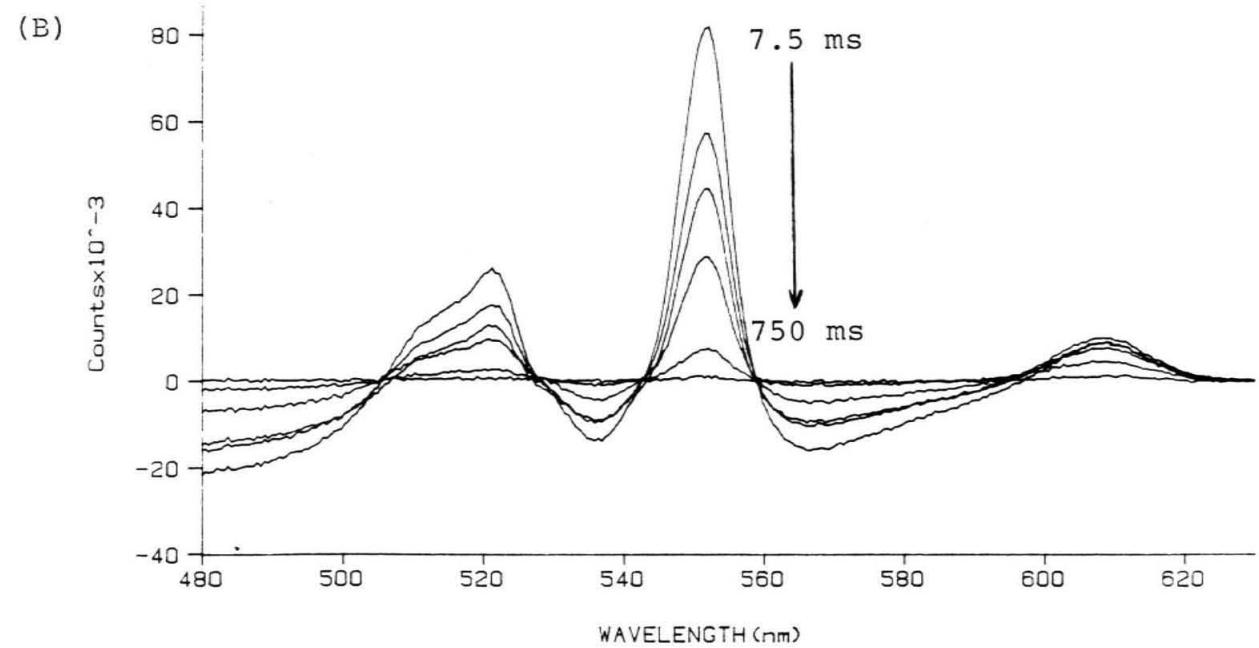

(B)
7.5 ms
750 ms
Countsx10^-3
WAVELENGTH (nm)

TABLE 1

Dependence of uncorrected stopped-flow fluorescence change on concentration.

Sample	Fluorescence change
2.5 μM cytochrome oxidase + 13.8 μM ferrocytochrome *c* [§]	+ 3.8 %[#]
1.5 μM cytochrome oxidase + 13.8 μM ferrocytochrome *c*	+ 3.8 ± 0.25 %
1.5 μM cytochrome oxidase + 9.7 μM ferrocytochrome *c*	+ 2.35 ± 0.25 %

[§]Concentrations before 1:1 mixing in stopped-flow apparatus.

[#]Excitation at 280 nm, slit width 4.

TABLE 2

Uncorrected stopped-flow fluorescence changes and time constants[§]

Sample	Excitation at 280 nm	Excitation at 304 nm
Cytochrome oxidase + cytochrome c^{2+}	+ 2.8% (2.9 s^{-1})	+ 13.5% (3.0 s^{-1})
Cytochrome oxidase + cytochrome c^{3+}	– 0.3% (0.5 s^{-1})	+ 2.6% (4.3 s^{-1})
Cytochrome oxidase + buffer	– 0.7% (1.9 s^{-1})	+ 2.3% (2.3 s^{-1})
Trace cytochrome oxidase + cytochrome c^{2+}	*	+ 13.5% (0.54 s^{-1})
Trace cytochrome oxidase + cytochrome c^{2+}	*	+ 13.2% (0.013 s^{-1})
Buffer + buffer	*	– 0.5% (0.4 s^{-1})

[§]Experimental conditions: Slit width at 280 nm excitation = 4, slit width at 304 nm = 1.8. Syringes of the stopped flow fluorometer loaded with 13.8 μM cytochrome *c* , 2.5 μM cytochrome oxidase, except in trace oxidase conditions where a small aliquot of 2.5 μM was added to buffer. Further details in text.

*In the absence of cytochrome oxidase, excitation at 280 nm produced very weak signals with unusable signal to noise ratios.

cytochrome *c* oxidation and provides enough fluorescence intensity for the inner filter effect to be visible. The fluorescence change depends in both rate and magnitude on the oxidation of cytochrome *c*.

A significant increase in the extent of the fluorescence rise was observed as the excitation wavelength was increased from 280 nm to 304 nm. This parallels an increasing absorption difference spectrum of reduced minus oxidized cytochrome *c*. Due to sample geometry, the inner filter effect at the excitation wavelength is larger than the effect at the emission wavelength, but both are observable. The region of the emission spectrum collected by the photomultiplier is divided almost equally into a part that has decreasing optical absorption during the reaction (less than 339 nm, see Figure 11A) and a part that has increasing optical absorption (more than 339 nm). The emission beam can be filtered to selectively view these regions which have opposite inner filter effects. When a 313 ± 20 nm bandpass filter is used, the fluorescence change increases to 3.9% for excitation at 280 nm (up from 2.8%). When a highpass filter with cutoff of 335 nm is used, the fluorescence change drops to 1.8%, and when this filter is doubled, raising the cutoff to about 345 nm, the fluorescence change becomes 1.2%.

Although the inner filter effect of cytochrome *c* dominates the stopped-flow fluorescence time course, two other effects are observable. When a sample of cytochrome oxidase and cytochrome *c* is illuminated at 280 nm or 288 nm, the fluorescence appears to decrease slowly, dropping by 5 – 6 % in ten minutes. The nearly linear decay of fluorescence, displayed in Figure 12, does not depend on the presence of cytochrome *c*, but occurs even if cytochrome oxidase is mixed with buffer. This drop in fluorescence is minimal (< 0.3%) when the sample is excited at 304 nm. Much of this fluorescence change can be attributed to inner filter effects from the slight increases in turbidity of the enzyme as it sits at room temperature. However, in a parallel experiment where oxidase was mixed with buffer or with cytochrome *c* by hand and observed for longer time periods, the changes in absorbance did not entirely explain the changes in fluorescence. The fluorescence time course of one such trial, shown in Figure 13, is extremely heterogeneous, but its coarse features are reproducible under identical mixing conditions.

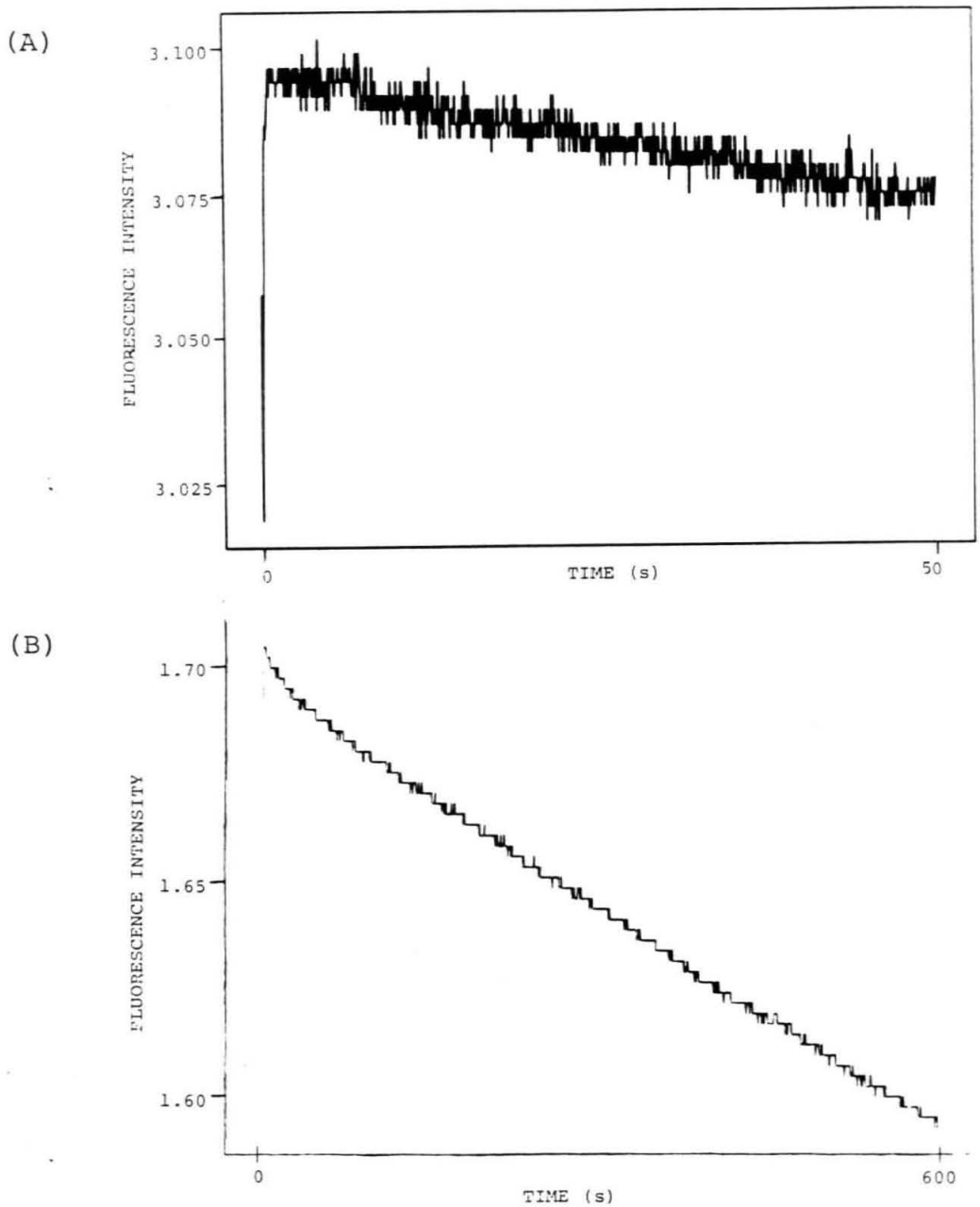


FIGURE 12. A. Fluorescence time course of the stopped flow mixing of 1.7 μM cytochrome oxidase with 13.8 μM cytochrome *c*. Excitation at 280 nm, slit width 4 nm. B. Fluorescence time course of the stopped flow mixing of 2.37 μM cytochrome oxidase with lauryl maltoside buffer. Excitation at 288 nm, slit width 2.8 nm.

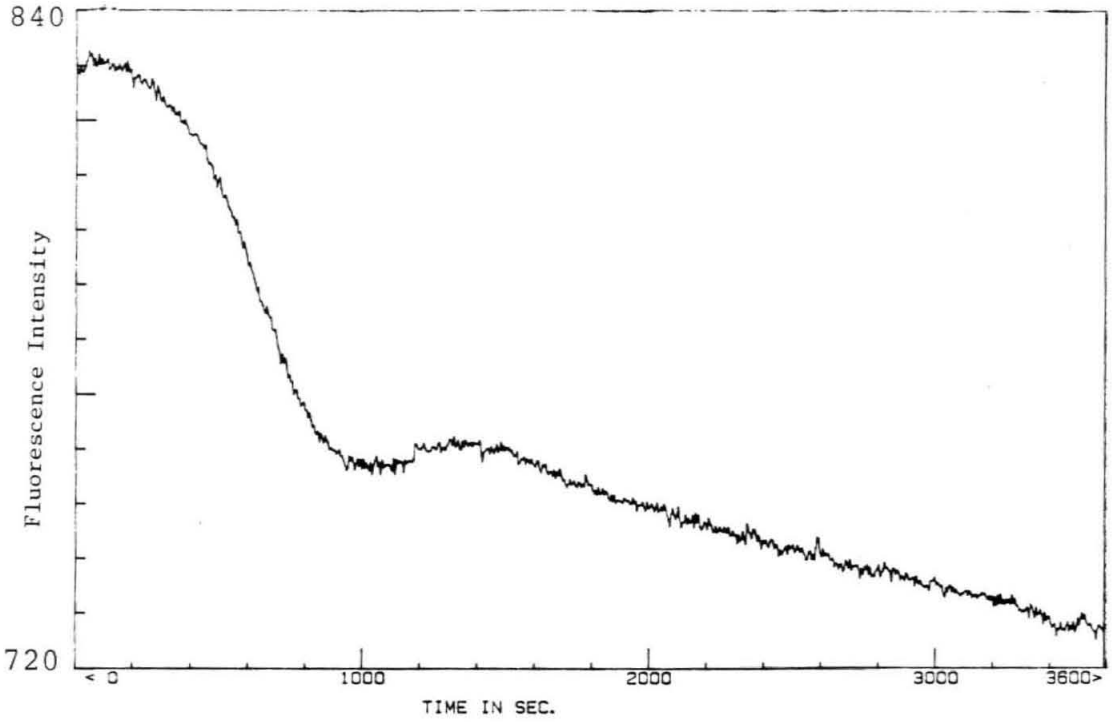


FIGURE 13. Fluorescence time course after mixing of equal volumes of 2.5 μM cytochrome oxidase and 13.8 μM cytochrome *c*. Excitation at 280 nm, slit width 8 nm. Emission at 330 nm, slit width 8 nm.

Whereas the fluorescence stops decreasing after 20 or 30 minutes, the absorbance of an identical sample increases steadily after an initial lag. Hill *et al.* (1988) have reported increases in the tryptophan fluorescence of cytochrome oxidase upon partial denaturation. Such increases in fluorescence could offset the inner filter effects due to turbidity and give rise to the type of complex behavior observed. The concentrations of oxidase used in this experiment are similar to those required for subunit III depletion by incubation in lauryl maltoside buffer (Hill & Robinson, 1986), so it is expected that the enzyme would partially denature during room temperature incubation. The observed increases in turbidity also point to protein degradation. It is sobering to realize that something as simple as doubling the detergent:protein ratio by mixing cytochrome oxidase with buffer can result in an apparent fluorescence change of 10% in less than half an hour.

The second minor effect on the stopped-flow fluorescence time course, apparent in Table 2, is observed when cytochrome oxidase is mixed with buffer and excited at 304 nm. A 2 – 3 % increase in fluorescence occurs on a similar time scale as the large cytochrome *c* inner filter effect. This effect is not seen when the sample is excited at 280 nm. One possible explanation is that the fluorescence of the lauryl maltoside is affected by changes in aggregation brought on by a change in the detergent:protein ratio.

It is clear that tryptophan fluorescence studies of protein conformations need to be accompanied by careful optical studies and other controls. A cursory application of inner filter corrections is not enough to prevent misinterpretation of data. Furthermore, actual changes in fluorescence may be due to general protein denaturation instead of catalytic conformational transitions. The fluorescence changes observed in this study appear to be linked to the structural degradation of cytochrome oxidase rather than to redox-linked conformational transitions.

The possibility remains that conformational transitions of cytochrome oxidase might produce changes in the tryptophan fluorescence. If the unloaded/loaded transition is linked to oxygen binding at Fe_{a3}, then both the oxidized and reduced forms of the enzyme are in the unloaded conformation. The binding of cyanide to Fe_{a3}, however, does not change the fluorescence spectrum significantly, so there is little reason to expect that the

unloaded/loaded transition is observable by fluorescence spectroscopy.

Li (1990) has reported transient changes in cytochrome oxidase fluorescence when either the fully reduced carbon monoxide inhibited (COFR) or mixed valence carbon monoxide inhibited (COMV) enzyme is photolyzed in the presence of oxygen. A rapid (faster than 10^6 s^{-1} for COFR and faster than 10^5 s^{-1} for COMV) increase of 2 – 5 % in the fluorescence is observed, followed by a slower (80 s^{-1} for COFR and 110 s^{-1} for COMV) decay to the original value. This transient change in fluorescence does not occur in the absence of oxygen and therefore is not due to carbon monoxide photolysis and recombination. The rapid increase in fluorescence is on a time scale consistent with oxygen binding to Fe_{a3}, but it is unclear what process occurs on the millisecond time scale in both the COFR and COMV forms of the enzyme and could account for the decrease in fluorescence. The COFR enzyme should rapidly form the pulsed enzyme and be in the unloaded state, whereas the COMV enzyme is trapped at Compound C, in the loaded state. Therefore it is unlikely that the change in fluorescence reflects any of the conformational transitions involved in proton pumping. Furthermore, parallel optical studies at both the excitation and emission wavelengths are needed to determine whether the observed change in fluorescence is due to changes in optical absorptions or actual changes in fluorescence. Even if the fluorescence changes are second order effects of absorption changes, it is clear that some type of conformational transition or reaction is occurring.

Two types of conformational transitions are expected during proton pumping in cytochrome oxidase: a switching transition between loaded and unloaded forms of the enzyme which is allosterically controlled by the binding of intermediates at the binuclear site and a gating transition which is linked to the redox state of the coupling site. Neither type of conformational transition has yet been accessible to direct experimental measurement. The use of tryptophan fluorescence spectroscopy to probe conformational changes relevant to proton pumping has not proved promising.

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