Cytochrome c Oxidase : Studies of Electron Input and Intramolecular Electron Transfer

Thesis by

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Abstract

Cytochrome c oxidase is the terminal enzyme of the electron transport chain in mitochondria. This enzyme catalyzes the transfer of electrons from ferrocytochrome c to dioxygen and reduces it to water. Concomitant with electron transfer and dioxygen reduction, cytochrome c oxidase pumps protons from the matrix side to the cytosolic side of the inner membrane, contributing to the formation of a transmembrane electrochemical gradient. As such electron transfer plays a central role in the function of the enzyme. This thesis investigated the input of electrons into the enzyme, the flow of electrons among redox-active metal centers in the enzyme as well as conformational changes associated with the redox state changes of the protein.

During electron transport, cytochrome c diffuses to a binding site or sites on cytochrome c oxidase. Extensive chemical modification studies have indicated that seven highly conserved lysine amino acids surrounding the heme crevice of cytochrome c are involved in the formation of an electrostatic complex with cytochrome c oxidase. Lysine 86 in cytochrome c is believed to be crucial in the binding of cytochrome c with cytochrome c oxidase. In order to study the effect of binding orientation between cytochrome c and cytochrome c oxidase with respect to the intracomplex electron transfer, we modified cytochrome c with ruthenium bis(bipyridine) dicarboxybipyridine at lysine 86 (Ru-86-cytochrome c). Our results showed that upon laser excitation of the preformed complex of Ru-86-cytochrome c and cytochrome c oxidase, electron was transferred rapidly from the ruthenium group to the ferric heme of cytochrome c; subsequently to the

cytochrome c oxidase. The observed intracomplex rate constants for the oxidation of cytochrome c are found to be biphasic with magnitudes of 560 s^{-1} for one phase and 114 s^{-1} for the other phase. The rate constant for the reduction of cytochrome a in cytochrome c oxidase is 2.3 x 10^4 s^{-1}. No reduction of Cu_A was observed at 830 nm. Although the observed rate constants for the oxidation of cytochrome c are slow, we believe there is a fast kinetic phase for this process beyond the resolution capability of the instrument. Apparently, the bulky ruthenated moiety on cytochrome c alters the binding orientation of cytochrome c with cytochrome c oxidase. As a result, cytochrome c preferentially transfers electron directly to cytochrome a rather than to Cu_A. The intracomplex electron transfer rate also exhibited ionic strength dependence as expected.

Internal electron transfer in cytochrome c oxidase was investigated by photolysis of CO-bound mixed-valence form of the enzyme. Upon CO photodissociation, ferrocytochrome a_3 was generated in less than 0.1 μsec, and a subset of the reduced cytochrome a_3 was reoxidized with biphasic rate constants of k_1 = 1.0 x 10^6 s^{-1} and k_2 = 7.8 x 10^4 s^{-1}. Reduction of cytochrome a was also observed with biphasic rate constants of k_1 = 1.6 x 10^6 s^{-1} and k_2 = 9 x 10^4 s^{-1}. The stoichiometry of oxidized cytochrome a_3 to reduced cytochrome a was found to be 1:1. No apparent electron transfer to Cu_A was observed at 830 nm. These results indicate that there is a significant electron reequilibration only between cytochrome a_3 and cytochrome a upon photodissociation of the CO-bound mixed-valence enzyme.

The nature of zinc in cytochrome c oxidase was investigated by depletion of zinc with mercuric chloride. The removal of zinc does not alter

the steady-state and transient electron transfer activities of the enzyme. The study indicates that zinc plays a structural role in the enzyme by serving as a bridge between subunit VIa and VIb. Based on indications that there is allosteric interaction companying redox state changes of cytochrome c oxidase, we probed the protein matrix for conformational changes using a fluorescence label on the enzyme. As a result of our findings, we concluded that there is a redox-linked conformational change in cytochrome c oxidase.

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Abbreviations

1,5-I-AEDANS, N-iodoacetyl-N'-(5-sulfo-1-naphthyl)ethylene-diamine;

ATP, adenosine triphosphate;

bpy, bipyridine;

CcO, cytochrome c oxidase;

cyt c, cytochrome c;

CO, carbon monoxide;

3CP, 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy;

dcbpy, dicarboxy(bipyridine);

DCP-AES, direct current plasma atomic emission spectrometry;

5-DRF, 5-deazariboflavin;

DMF, N,N-Dimethylformamide;

EDTA, ethylenediamine tetraacetic acid;

ENDOR, electron nuclear double resonance;

EPR. electron paramagnetic resonance;

EXAFS, extended x-ray absorption fine structure;

FMA, fluorescein mercuric acetate;

k_{app}, apparent rate constant;

NCD-4, N-cyclohexy-N'-(4-dimethylaminonaphthyl)carbodiimide;

*p*HMB, *p*-(hydroxymercuri)benzoate;

SAS PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis;

Tris, tris(hydroxyethyl)aminomethane;

μsec, micro second;

I Introduction

Cytochrome c oxidase is the oxygen-activating enzyme in the mitochondria respiratory chain of eukaryotes as well as certain prokaryotes. It exists in a wide variety of aerobic organisms, including animals, plants, yeast cells, bacteria, fungi and single-celled eukaryotes. The essential nature of cytochrome c oxidase may be exemplified by the fact that it enables cells to live on a large energetic advantage. It has been estimated that nearly 90% of the O_2 consumed by living organisms on earth are involved in the dioxygen chemistry of cytochrome c oxidase. The very critical dependence of vital organs, including brain, heart muscle and kidney on aerobic metabolism is another facet of cytochrome c oxidase's central role in physiology (Wikström et al., 1981).

The process of mitochondrial electron transfer ends with the reduction of dioxygen to water by cytochrome c oxidase. The reducing equivalents for the four-electron reduction of molecular oxygen to water are derived from ferrocytochrome c (cyt c): $4\text{cyt } c^{2+} + O_2 + 4H^+ \rightarrow 4\text{cyt } c^{3+} + 2H_2O$. The free energy derived from this reaction is converted into a proton electrochemical gradient across the inner mitochondrial membrane which ultimately drives the synthesis of ATP (Krab and Wikström, 1987). To accomplish this energetic coupling, the electrons enter the enzyme from the cytosolic side of the membrane, and the protons are consumed from the matrix side. In this manner, the membrane sideness is exploited to produce a charge separation and a proton gradient across the membrane.

The electrogenic proton pumping process is coupled to the transmembrane electron transfer. As four electrons flow through the enzyme, cytochrome c oxidase also pumps up to four protons from the

mitochondrial matrix to the cytosol for every dioxygen molecule being reduced. Thus, the stoichiometry of the overall proton pumping process can be as high as one proton per electron. Thermodynamic considerations expect that proton pumping can only be coupled with the last two electron transfer during the four electron transfer process. Experimental results have confirmed this hypothesis (Chan and Li, 1990; Wikström, 1989; Babcock and Wikström, 1992). For each of the last two electrons transfers through the cytochrome c oxidase, two protons are pumped up, resulting in a stoichiometry of $2H^+/e^-$ for the dioxygen reduction cycle.

Cytochrome c oxidase has a complicated composition and structure. The enzymes from eukaryotic organisms are made up of as many as 12-13 different polypeptides. Even those of simple bacteria have 2 or 3 subunits (Kadenbach et al., 1983). Most of these subunits are encoded in nuclear genes and synthesized inside the cytoplasm, but the three largest subunits (I, II, and III) are encoded in the mitochondrial genome and synthesized inside the mitochondria. The three "mitochondrial" subunits appear to make up the functional core of the enzyme. And all of the redox-active metal centers are believed to reside in subunit I and II (Wikström, 1981; Chan and Li, 1990).

The molecular events catalyzed by cytochrome c oxidase are mediated by four redox-active metal centers, two coppers and two heme irons. One iron and one copper, cytochrome a_3 and copper B (Cu_B), form a binuclear cluster where dioxygen is bound and reduced. The other two centers, cytochrome a and copper A (Cu_A), act as electron acceptor from ferrocytochrome c and transfer the electrons to the dioxygen binding site (Blair et al., 1983). It has been widely speculated that either the Cu_A site or

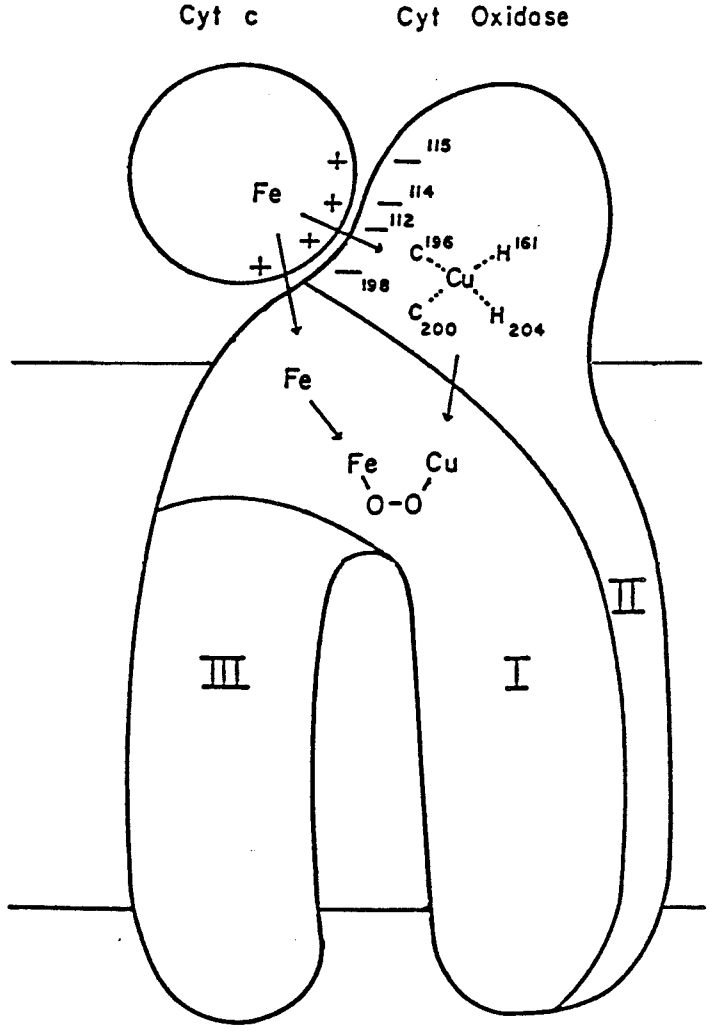
cytochrome a or recently, the binuclear center is the coupling site of electron transfer to proton pumping (Wikström and Casey, 1985).

In mammals, cytochrome c oxidase is an integral membrane protein existing in the inner membrane of the mitochondrion. The isolated beef heart cytochrome c oxidase is a dimer, but the functional unit appears to be a monomer. Electron microscopy and image reconstruction analysis of two-dimensional crystals of beef heart cytochrome c oxidase have resulted in a low resolution picture of the enzyme structure, thus providing a framework for the understanding of the enzyme topology, as well as its position in the lipid bilayer (Deatherage et al., 1982). As shown in Figure I-1, the enzyme complex spans the lipid bilayer and is arranged as three domains. One of the domains extends into the cytosolic side. Each of the other two traverses the membrane and extends a small way from the matrix side. The molecular weight of cytochrome c oxidase ranges from 70 to 120 kDa (Brunori et al., 1987). So far there are uncertainties regarding the subunits assembly and the minimal molecular weight of the enzyme. Studies of cytochrome c oxidase address several important questions in biology including the mechanisms of energy coupling, communication between the mitochondrial and nuclear genomes, and assembly of organelles. Therefore, an understanding of the structure and function of cytochrome c oxidase at the fundamental and molecular level is a must.

METAL CENTERS

It is generally accepted that eukaryotic cytochrome c oxidase contains four redox-active centers, which are responsible for its catalytic activity. These four groups can be distinguished on the basis of their function. Since there is

Figure I-1: A schematic diagram of cytochrome c oxidase in the inner mitochondrial membrane, including the approximate relative positions of the redox-active metal centers. The carboxylates involved in binding with cytochrome c are shown.


Cyt c
Cyt Oxidase
Fe
115
114
112
198
C 196
H 161
Cu
C 200
H 204
Fe
Fe
Cu
O-O
III
I
II

no high resolution crystal structure available for cytochrome c oxidase, the present identification of metal center ligands and structures, the measurement of distances between metal centers, have come from the spectroscopic studies. Some additional information about the metal center structures have also come from amino acid sequence homology data. It is now generally agreed that all the four redox-active metal centers are located in subunit I and II (Mueller et al., 1988). Specifically, cytochrome a, cytochrome a_3, and Cu_B locate in subunit I (Winter et al., 1980; Ludwig, 1980). Cu_A resides in subunit II (Holm et al., 1987; Martin et al., 1988; Hall et al., 1988).

Recent metal composition studies of cytochrome c oxidase from beef heart source and certain other tissues have indicated that zinc, magnesium, and additional copper (in addition to Cu_A and Cu_B) are also tightly bound to the enzyme (Einarsdottir and Caughey, 1985; Pan et al., 1991ab; Lin et al., 1993). However, for quite a long time, the nature and the subunit location of these recently discovered metal centers remain unknown. These metal centers do not exhibit spectroscopic signature, neither do they appear to be redox-active. They are difficult to be removed without denaturing the protein.

Ligands to Metal Centers All four redox-active metal centers are structurally and spectroscopically distinct. Both irons exist as hemes A which bear a formyl group and a long isoprenoid chain on its periphery.

Cytochrome a is a six-coordinate low-spin heme A with both of its axial ligands being histidines (Babcock and Callahan, 1983; Martin et al., 1985). EPR and isotope incorporation studies have indicated that one of the axial ligands

to cytochrome $\underline{a}_3$ is a histidine, the other axial position is part of the catalytic site for dioxygen reduction.

Cu_B is UV-visible and EPR silent in most states of the enzyme. It is known from EXAFS and ENDOR experiments, that in the oxidized state, Cu_B has three nitrogen bearing ligands, of which are probably histidines, and one heavier ligand (S or Cl) (Li et al., 1987). Cooperating with cytochrome $\underline{a}_3$, Cu_B makes up part of the catalytic site for dioxygen binding and reduction. The two metal ions form a binuclear center and are antiferromagnetically coupled through a bridging ligand (Brudvig et al., 1986; Scott et al., 1988). After one turnover, this ligand is displaced, yielding a much more active "pulsed" oxidase (Brudvig et al., 1981; Colosimo et al., 1981; Armstrong et al., 1983). A variety of external ligands can be coordinated to the binuclear center, including F^-, CN^-, formate, and peroxide in the oxidized state and O_2, CO, and NO in the reduced state.

Cu_A is a spectroscopically unique biological metal center. EXAFS studies of native and chemically modified cytochrome $\underline{c}$ oxidase have suggested that two cysteine sulfurs are ligands of Cu_A (Li et al., 1987). Spectroscopic and amino acid sequence data are consistent with a structure in which Cu_A is ligated by two cysteine residues in subunit II (Steffens et al., 1987; Hall et al., 1988). EPR and ENDOR experiments implicate that at least one histidine and at least one cysteine are ligands to Cu_A (Stevens et al., 1982; Martin et al., 1988). Accordingly, Chan and co-workers have proposed that Cu_A is ligated by two histidine imidazole nitrogen atoms and two thiolate sulfurs from cysteine residues (Chan and Li, 1990). It has also been suggested

that the presence of a second cysteine makes Cu_A spectroscopically unique and distinct from those of type 1 Cu^{2+} in blue copper proteins (Malmström, 1990).

The role of Cu_A in cytochrome c oxidase has attracted considerable attentions for a long time. Numerous experimental evidence have indicated that Cu_A plays an important role in the function of cytochrome c oxidase. It has been reported that Cu_A is the metal cofactor most exposed to the cytosolic side of the membrane. From the carbodiimide modification study, it is noted that Cu_A binding site in cytochrome c oxidase is close to negatively charged carboxyl residues on subunit II that are involved in the docking of cytochrome c (Millett et al., 1983).

In addition to the four redox-active metal centers, it is now established that zinc ion and magnesium ion as well as extra copper (designated as Cu_X) are intrinsic components of cytochrome c oxidase. A stoichiometry of 5Cu/4Fe/2Zn/2Mg/ per cytochrome c oxidase dimer has been proposed and confirmed (Einarsdottir and Caughey, 1985; Pan et al., 1991a).

Work presented in this thesis has shown that Zn ion can be completely dissociated from cytochrome c oxidase without denaturing the protein. In the enzyme, zinc ion plays a structural role by providing a bridge between subunit VIa and VIb. Cysteine sulfurs are the most likely ligands for zinc (Naqui et al., 1988; Scott, 1989). Electron transfer activity assay indicates that zinc does not appear to involve in electron transfer in cytochrome c oxidase.

Chan and co-workers have confirmed the suggestion of Yewey et al. (1987) by NCD-4 labeling that magnesium of cytochrome c oxidase is located

in subunit IV (Lin et al., 1993). Sequence comparison suggests that Glu-136 in subunit IV of cytochrome c oxidase is the most likely carboxyl ligand for the magnesium ion. The functional role of magnesium in mamalian cytochrome c oxidase might be to provide an ATP binding site in association with subunit IV (Yewey and Caughey, 1987).

The existence of Cu_X as an intrinsic metal component in cytochrome c oxidase has been confirmed. Cu_X is removable by either monomerization of the enzyme or subunit III depletion. It has been proposed that Cu_X is associated with subunit III and plays a structural role in the dimerization of cytochrome c oxidase, functioning as a bridge between the two subunit III polypeptides within the dimer (Pan et al., 1991a).

Intermetal Distances Most of the information regarding the distances between the metal centers have been derived from spectroscopic studies.

1) cytochrome a to Cu_A It has been reported that EPR saturation properties of Cu_A and its ENDOR spectrum depend on the reduction/oxidation states of cytochrome a (Brudvig et al., 1984; Scholes et al., 1984). Based upon these observations and measurements, Brudvig et al. (1984) calculated a distance of 13 to 26 Å between cytochrome a and Cu_A. Later Goodman and Leigh (1985) reported a distance between 8 and 13 Å.

2) cytochrome a to cytochrome a_3 Goodman and Leigh (1987) reported a distance of 19 $\pm$ 8 Å between cytochrome a and cytochrome a_3 based on their electron spin relaxation rate measurements in the azide-bound

compound of cytochrome c oxidase. Brudvig et al. (1984) calculated a distance of 20 Å from a reinterpretation of earlier EPR studies of the nitrosyl adduct of ferrocytochrome a_3. Capaldi (1990) mentioned a distance of 12-16 Å in his review paper.

3) cytochrome a_3 to Cu_B The distance between cytochrome a_3 and Cu_B is the best characterized intermetal distance in cytochrome c oxidase. The oxidized enzyme reacts with azide and NO to produce species containing either ferrocytochrome a_3-N_3 or ferrocytochrome a_3-NO, and Cu_B^{2+} at the dioxygen binding site. Magnetic interaction between the two spins gives rise to a triplet signal, from which a distance of 3.4 Å between "spin centers" was calculated (Stevens et al., 1979; Blair et al., 1983). A value of 3.8 Å for the distance between the iron of cytochrome a_3 to Cu_B was calculated from EXAFS measurements by Powers et al. (1979).

Redox Potentials The redox potential of cytochrome c is 260 mV (vs. NHE) in solution (Taniguchi et al., 1982) and is reported to decrease to as low as 220 mV when bound to cytochrome c oxidase (Schroedl and Hartzell, 1977). The redox potential for the reduction of dioxygen to water has been measured to be 810 mV (Wikström et al., 1981). This span defines the range of potentials over which cytochrome c oxidase operates, as well as the amount of free energy available to be released. It is generally assumed that the largest free energy change during enzymatic reduction of dioxygen is associated with the electron transfer step from the primary acceptors (Cu_A and cytochrome a) to the dioxygen intermediates anchored at the dioxygen reduction site. In the native form of cytochrome c oxidase, the redox potential of cytochrome a is fairly high, about 350 mV. However, the high redox potential decreases to

about 280 mV when the metal ions at dioxygen binding site are reduced. The redox potential of cytochrome a also shows a pH dependence of 30 mV/pH unit (Aratzabanov et al., 1978). But the pH dependence appears to be dependent on the state of the enzyme. For example, in the CO-bound mixed-valence form of cytochrome c oxidase, the pH dependence is only about 9 mV/pH unit (Blair et al., 1986). Compared with Cu_B, Cu_A has lower redox potential of about 280 mV. The redox potential of cytochrome a_3 is about 380 mV when dioxygen is absent. Due to the lack of appropriate spectroscopic signature, the redox potential of Cu_B was reported to be about 340 mV from an indirect measurement (Lindsay, 1975). Under physiological condition, the effective redox potentials of cytochrome a_3 and Cu_B will be much higher due to the involvement of dioxygen chemistry at these two metal centers.

Interaction Between The Redox-Active Metal Centers Considerable experimental evidence have indicated that there are strong redox interactions between the metal centers in cytochrome c oxidase. The redox potentials of the metal centers can vary in response to the redox states of other metal centers in the enzyme (Goodman, 1984; Blair et al., 1986). The interaction between cytochrome a and Cu_A has been studied by spectroelectrochemical techniques (Blair et al., 1986a) and reported to be an anticooperative interaction of ~40 mV, which means that when cytochrome a is reduced, the redox potential of Cu_A is lowered by 40 mV. This interaction was also observed by ENDOR measurements (Fan et al., 1988). It was suggested that the reduction of cytochrome a affects the interaction of oxidized Cu_A with its cysteine residue ligand. In addition, there are anticooperative interactions between cytochrome a and cytochrome a_3 (-35 mV), cytochrome a and Cu_B (-35 mV), and cytochrome a_3 and Cu_B (-35 mV). The results are summarized in

Figure I-2: Redox interactions between the redox-active metal centers of cytochrome c oxidase.

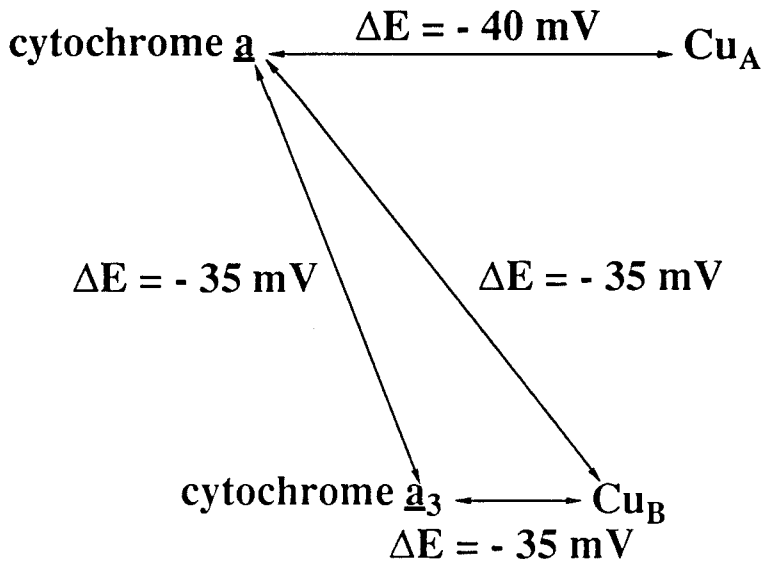
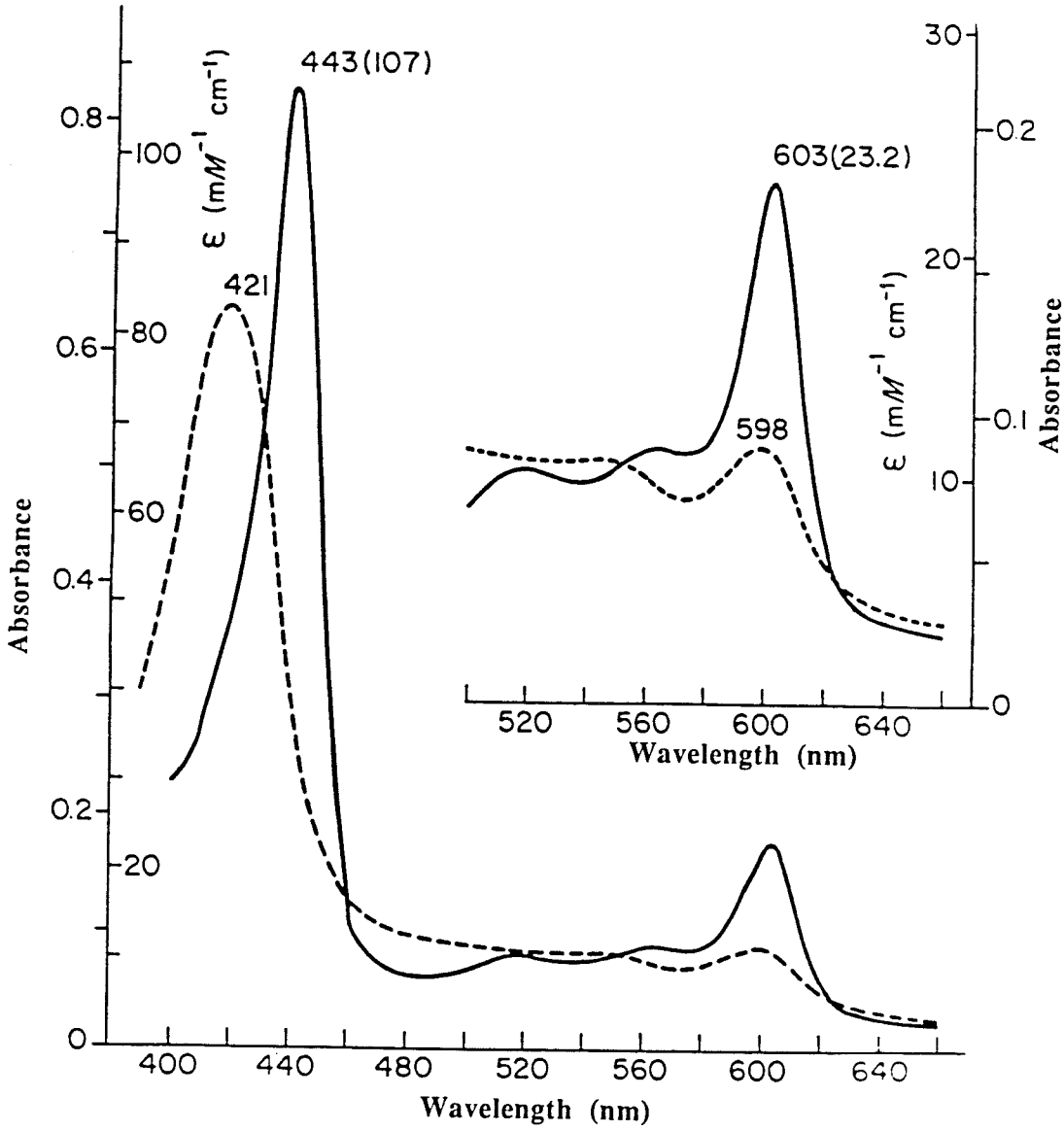

cytochrome a
ΔE = - 40 mV
Cu$_A$
ΔE = - 35 mV
ΔE = - 35 mV
cytochrome a$_3$
Cu$_B$
ΔE = - 35 mV

Figure I-2. The data in Figure I-2 illustrate that there is an anticooperativity between all the metal sites, except that Cu_A does not interact directly with the binuclear metal center. Blair et al. (1982) have suggested that there may be a similar redox interaction between the Cu_A site and cytochrome a_3 and/or Cu_B. More recently, it was noted that some anticooperative redox interactions do exist between Cu_A and the binuclear center for two samples of modified cytochrome c oxidase (Li et al., 1991). The interactions among the metal centers can be best explained by a mechanism in which these sites communicate via a protein scaffolding (Wang et al., 1986). The redox potentials given above are all "upper asymptotic" potentials, meaning that these are the potentials when all of the other metal sites are oxidized.

Optical Spectra Optical spectroscopic methods have proved to be very useful in the studies of cytochrome c oxidase. The absorption bands in the visible spectra of both the reduced and oxidized enzymes are mainly attributable to heme-associated transitions. Figure I-3 shows the visible spectra of the reduced and oxidized cytochrome c oxidase. In addition to the bands shown, the fully oxidized cytochrome c oxidase exhibits a band at 830 nm, which at least 85% is due to certain transition within Cu_A. This absorption band has been assigned to a metal-to-ligand charge-transfer transition between Cu_A^{2+} and a sulfur ligand (Beinert et al., 1962). The optical absorption of cytochrome c oxidase at 830 nm is a unique spectroscopic signature of Cu_A^{2+}. However, this absorption band is weak and broad with an extinction coefficient of 2.0 $mM^{-1}cm^{-1}$ (Blair et al., 1983).

Interpretation of the spectra shown in Figure I-3 in terms of contribution from cytochrome a and cytochrome a_3 has been a subject of

Figure I-3: Absorption spectra of fully reduced (——) and fully oxidized (- - -) cytochrome c oxidase. Extinction coefficients are on the cytochrome a basis. (From Wikström et al., 1981).


443(107)
421
603(23.2)
598
Absorbance
ε (mM⁻¹ cm⁻¹)
Wavelength (nm)
0
0.2
0.4
0.6
0.8
20
60
80
100
400
440
480
520
560
600
640
10
30
0.1

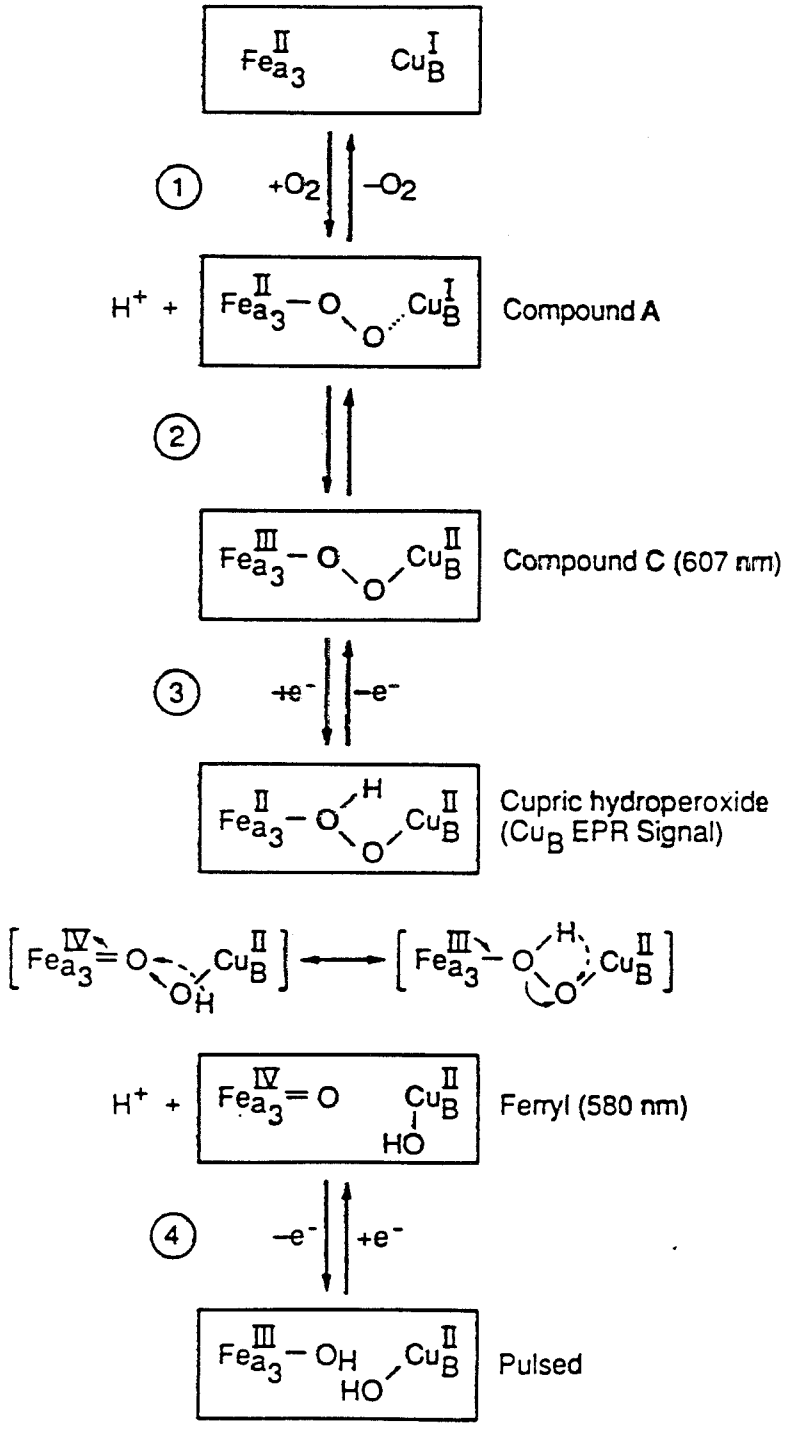
much controversy and ambiguity. At one time, the interactions observed in redox titrations were attributed to spectral interactions; that is, to the influence of the redox state of one site on the visible extinction coefficient of another site (Wikström et al., 1981). Such spectral interactions do exist. For example, Blair et al. (1982) have shown that there is a modulation of cytochrome $\underline{a}_3$ absorptions by a redox state change of Cu_B. However, a number of reports have argued that spectral interactions are small compared to redox interactions (Goodman et al., 1984; Blair et al., 1986; Ellis et al., 1986; Wang et al., 1986).

The classical method of evaluating the individual spectra of the two hemes is to make use of the fact that one heme but not the other binds ligands in both the ferrous and ferric forms. Using this "ligand technique," Vanneste (1966) reported a deconvolution of the visible spectra into contributions from cytochrome $\underline{a}$ and cytochrome $\underline{a}_3$. According to this source, the contributions from cytochrome $\underline{a}$ and cytochrome $\underline{a}_3$ to the reduced-minus-oxidized absorption difference at 444 nm are 45% and 55%, respectively; while the contributions at 605 nm are about 80% and 20%, respectively.

DIOXYGEN CHEMISTRY

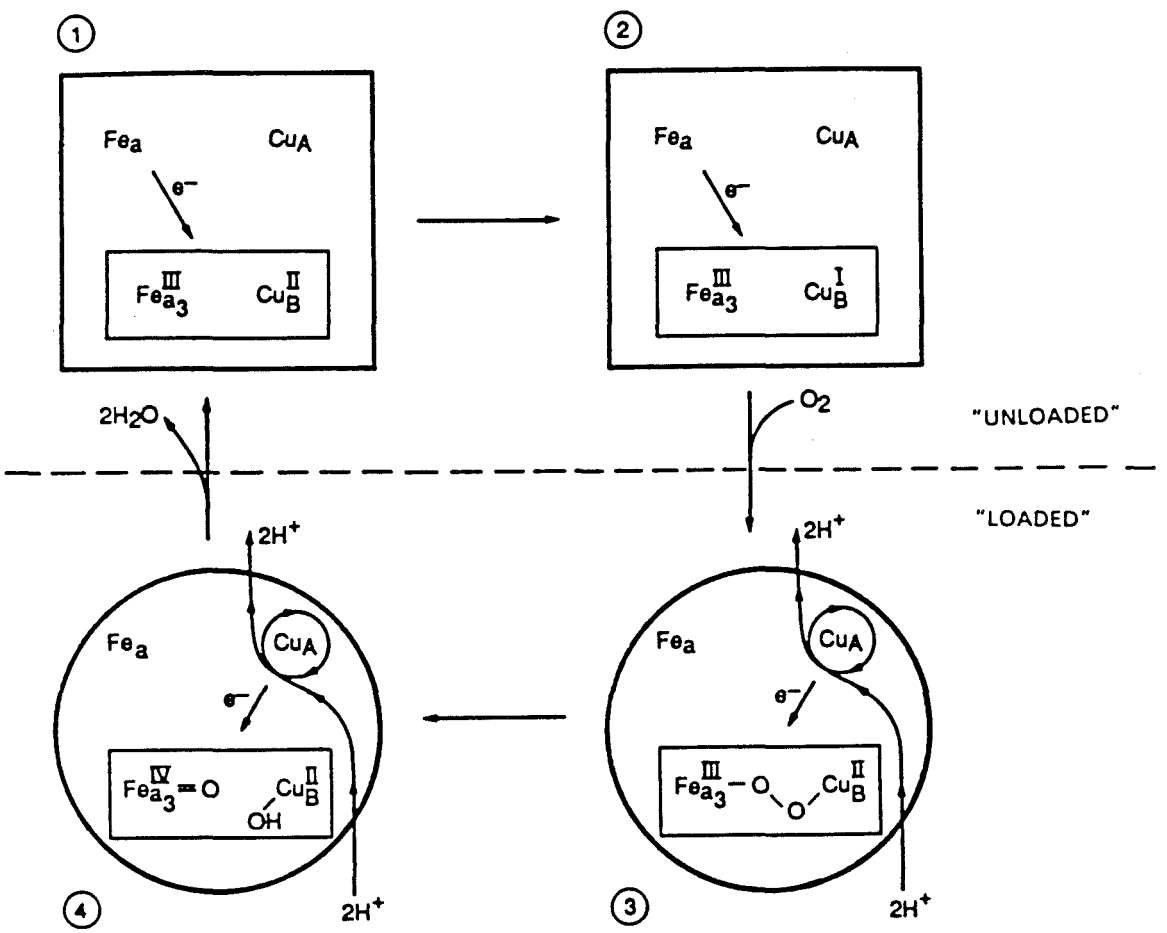
The current understanding of dioxygen reduction mechanism in cytochrome $\underline{c}$ oxidase is illustrated in Figure I-4 (Chan and Li, 1990). Dioxygen binds to the reduced binuclear center, forming a peroxide adduct, compound C. As two more electrons are transferred from the low potential metal centers sequentially, first the O-O bond is broken, forming the ferryl compound, and finally yielding the pulsed enzyme. The transfer of two more electrons from

Figure I-4: Scheme depicting the reduction of dioxygen at the binuclear site of cytochrome c oxidase. The four reduction steps of dioxygen are numbered and the electron transfer steps from the low potential metal centers are also indicated. (From Chan and Li, 1990).


Fe a3 II Cu B I
1
+O2
−O2
H+ +
Fe a3 II − O O Cu B I
Compound A
2
Fe a3 III − O O Cu B II
Compound C (607 nm)
3
+e-
−e-
Fe a3 II − O H O Cu B II
Cupric hydroperoxide
(Cu B EPR Signal)
Fe a3 IV = O O H Cu B II
Fe a3 III − O H O Cu B II
H+ +
Fe a3 IV = O Cu B II HO
Ferryl (580 nm)
4
−e-
+e-
Fe a3 III − OH HO Cu B II
Pulsed

the low potential centers to the high potential center, i.e. the binuclear site (not shown in Figure I-4) completes the catalytic cycle and prepares the enzyme to bind to dioxygen again. The pumping of two protons is coupled to the reduction of compound C to the ferryl intermediate, and another two protons are then pumped when the ferryl species is reduced to form the pulsed enzyme (Wikström, 1989). The electrons transferred to the "unloaded" binuclear center are not coupled to proton translocation (Figure I-5). When the catalytic cycle is divided into four steps on the basis of the four electrons transferred from the low potential metal centers to the high potential center, only the steps associated with the last two electron transfer are strongly exothermic under physiological conditions (Wikström, 1986; 1988). The irreversible step of the catalytic cycle includes the second unloaded electron being transferred to the binuclear site, the subsequent binding of dioxygen to the center, and the two electron reduction of dioxygen to compound C. The existence of both coupled and uncoupled electron transfers from the low potential metal centers to the dioxygen binding site requires a conformational switch between a pumping form of the enzyme and a non-pumping form. This can be accomplished either by changing the overall electron transfer route or by disengaging and engaging a coupling mechanism along the same electron transfer route. The irreversible step of the catalytic cycle traps cytochrome c oxidase in the loaded conformation. In itself, the unloaded to loaded conformational transition might be thermodynamically unfavorable, but it could also be linked to a downhill process such as dioxygen binding.

Figure I-5: A proposal for the mechanism of the turnover cycle of cytochrome c oxidase. (From Chan and Li, 1990).


1
2
Fea
CuA
e⁻
Fe$^{III}_{a_3}$
Cu$^{II}_{B}$
Cu$^{I}_{B}$
2H2O
O2
"UNLOADED"
"LOADED"
2H+
Fe$^{IV}_{a_3}$=O
OH
Fe$^{III}_{a_3}$–O–O–Cu$^{II}_{B}$
3
4

KINETICS

Cytochrome c oxidase catalyzes the reduction of dioxygen to water concomitant with the oxidation of ferrocytochrome c and proton translocation. During this process, electron transfer activity is essential to the function of the enzyme. A number of studies have implicated cytochrome a or Cu_A as site of redox-linkage for proton pumping. The free energy for electron transfer from either of these sites to cytochrome a_3 and Cu_B is large enough to drive the proton pumping reaction. Cytochrome a has been suggested to be involved in proton pumping because its redox potential is pH-dependent. On the other hand, Gelles et al. (1986) proposed a model in which redox-linked Cu_A ligand substitution reactions are coupled to proton pumping. Perturbation of the Cu_A site by p-Hydroxymercuribenzoate or heat treatment was found to inhibit proton pumping of cytochrome c oxidase, providing evidence to support this proposal (Nilsson et al., 1988; Li et al., 1988). It is also believed that intramolecular electron transfer from either cytochrome a or Cu_A to the dioxygen intermediates is coupled with the proton pumping activity of cytochrome c oxidase. Therefore, it is of particular interest to understand the detail mechanism of electron transfer in cytochrome c oxidase.

Two classes of technique have been employed to study the kinetics of electron transfer of the enzyme. One is the steady-state kinetic measurements, in which the rate-limiting step and events toward steady-state are studied. The second is transient kinetic investigation, which is designed to follow the electron transfer process in real time scale.

Steady-State Kinetics Under certain experimental conditions, cytochrome c oxidase can catalyze the oxidation of 30-600 molecules of ferrocytochrome c per second. Numerous works have been devoted to the study of this oxidation at steady-state by classical enzymatic kinetic methods. The oxidation of ferrocytochrome c can be monitored spectrophotometrically (Smith, 1955) since there are tremendous differences in the absorption spectra of the reduced and oxidized enzymes. In addition, the oxidation of cytochrome c is also accompanied with the reduction of dioxygen to water. The consumption of dioxygen can be monitored polarographically by means of the oxygen electrode (Ferguson-Miller et al., 1976).

The steady-state oxidation of ferrocytochrome c by cytochrome c oxidase is quite complicated. When the concentration of cytochrome c is varied, two kinetic phases are typically observed, each with a different maximum turn over number (TN_{max}) and K_m. These values also depend on pH, ionic strength of the buffer, and the detergent used to solubilize the protein (Sinjorgo et al., 1986). Binding studies demonstrated that cytochrome c oxidase binds cytochrome c with two different affinities, which correlate fairly well with the K_m values of the two kinetic phases. This was interpreted in terms of two catalytically active cytochrome c binding sites on the oxidase (Ferguson-Miller et al., 1976). On the other hand, it has been suggested that there may be only one catalytic cytochrome c binding site, but there are two different conformations of cytochrome c oxidase towards accepting electrons (Malmström and Andréasson, 1985; Thörnström et al., 1988).

When electrons enter the cytochrome c oxidase from ferrocytochrome c, cytochrome a and Cu_A are the first metal centers to be reduced. From these

two centers, the electrons are passed through the enzyme to cytochrome a_3 and Cu_B where the reduction of dioxygen to water occurs. The initial reduction of cytochrome a and Cu_A is fast. In stopped-flow experiments, with or without dioxygen present, biphasic reduction is observed at both metal centers. Wilson et al. (1975) suggested that cytochrome a was the initial electron acceptor since they found that reduction of Cu_A lagged behind that of cytochrome a. In contrast, Antalis and Palmer (1982) observed that Cu_A and cytochrome a were reduced at the same rate. Therefore, it is not clear whether cytochrome a or Cu_A is the initial electron acceptor or which of these redox centers transfers electrons to the dioxygen binding site. In these experiments mentioned above, initial rates between 10^6 and 3×10^7 $M^{-1}s^{-1}$ have been measured for the reduction of cytochrome a, in a variety of different enzyme preparations.

Transient Kinetics Biological electron transfer reactions are fast and extremely efficient. Stopped-flow technique often fails to measure the rate of these ultrafast electron transfer reactions with the conventional rapid mixing method. It has been reported that the mixing event takes about 4 millisecond. With such an instrumental dead-time, most of protein dynamic kinetics have proceeded to completion. Only since laser-flash technique is developed, are reactions of many orders of magnitude faster possible to be measured.

Electron Input from Ferrocytochrome c to Cytochrome c Oxidase

Cytochrome c binding was proposed to be the rate-limiting step from stopped-flow kinetics studies of the reaction between cytochrome c and cytochrome c oxidase (Speck et al., 1984; Sinjorgo et al., 1984). This problem

has prevented a complete analysis of the mechanism by which cytochrome c transports electrons to cytochrome c oxidase. A number of approaches have been developed to measure electron transfer reactions between two proteins held in a 1:1 complex. Most of these techniques are fast and not limited by mixing. Since the reactions probed are in 1:1 protein complexes, rate limitation due to diffusion is no longer a major concern. Cusanovich and Tollin (1980) developed a technique which utilizes flavins to photochemically initiate electron transfer in redox complexes. Hazzard et al. (1987) used this technique to measure the intracomplex electron transfer reaction between cytochrome c and cytochrome c peroxidase for the first time. Pulse radiolysis has been used to measure the rate of intracomplex electron transfer in the cytochrome b_5 and cytochrome c complex, as well as the cytochrome c and plastocyanin complex (McLendon and Miller, 1985; Peerey and Kostic, 1989). Winkler et al. (1982) and Isied et al. (1982) introduced the use of tethered ruthenium complexes, specifically attaching a $Ru(NH_3)_5$ group to histidine 33 of cytochrome c. This strategy was extended by Pan et al. (1988) to individually label lysine residues of cytochrome c by ruthenium bis(bipyridine) dicarboxybipyridine. The Ru-cytochrome c derivatives have been found to have important applications in studies of electron transfer in protein-protein complexes (Pan et al., 1990; Pan et al., 1993).

A number of approaches have been exploited to study the reaction between ferrocytochrome c and cytochrome c oxidase. Hill (1991) carried out flash photolysis studies of a ferrocytochrome c and reduced, CO-inhibited cytochrome c oxidase complex in the presence of dioxygen. The data are consistent with electron input from cytochrome c to Cu_A with a rate constant greater than 70,000 s^{-1}, followed by electron transfer from Cu_A to cytochrome a

with a rate constant of 6000 s^{-1}. Using a different approach, Hazzard et al. (1991) employed flavin flash photolysis to measure an intracomplex rate constant of 630 s^{-1} for electron transfer from cytochrome c to cytochrome a in cytochrome c oxidase at low ionic strength. This rate was found to increase to a maximum value of 1470 s^{-1} at 110 mM ionic strength. Pan et al. (1988) developed a technique to label a ruthenium(II) complex specifically at a surface lysine residue of cytochrome c. The Ru(II) group can be photoexcited to a metal-to-ligand charge-transfer state, Ru(II)*, which is a strong reducing agent. This species then can transfer an electron to the heme of cytochrome c on a nanosecond time scale (Durham et al., 1989). Afterwards, the electron will be transferred to cytochrome c oxidase. Pan et al. (1993) reported that the ruthenium photoexcitation technique can be used to measure rapid intracomplex electron transfer between Ru-cytochrome c derivatives and cytochrome c oxidase. The mechanism for Ru-cytochrome c derivatives modified at lysines far from the heme crevice domain involves initial electron transfer to Cu_A with a rate constant greater than 10^5 s^{-1}. Cu_A then transfers an electron to cytochrome a with a rate constant of 2×10^4 s^{-1}. On the other hand, the derivatives in which the lysines in the heme crevice domain are modified react much more slowly, with rate constants for oxidation of ferrocytochrome c and reduction of cytochrome a ranging from 1000 to 6000 s^{-1}. No apparent reduction of Cu_A was observed. These results clearly indicate that the binding orientation between cytochrome c and cytochrome c oxidase is critical in determining the electron transfer pathway.

It has been generally agreed that Cu_A is the primary electron acceptor from ferrocytochrome c to the native cytochrome c oxidase. However, certain experimental evidence suggest that there may exist two electron input

pathways. Li et al. (1991) have reported that the Cu_A intrinsic reduction potential of p-Hydroxymercuribenzoate modified cytochrome c oxidase decreased by almost 150 mV. From thermodynamic consideration, however, direct electron transfer from ferrocytochrome c to cytochrome a seems more likely to be the electron input pathway. Pan et al. (1991c) found that for a Cu_A-depleted cytochrome c oxidase sample, cytochrome a can also accept an electron directly from ferrocytochrome c, albeit at a slower rate. This is the first direct evidence to support the possibility that there are distinct bi-pathway of electron input from ferrocytochrome c to cytochrome c oxidase.

Intramolecular Electron Transfer in Cytochrome c Oxidase

Following the entry into the enzyme at Cu_A and/or cytochrome a, electrons are transferred to the cytochrome a_3 and Cu_B binuclear center intramolecularly. In order to study these reactions, a technique known as "flow flash" has been developed (Gibson and Greenwood, 1963). In the flow-flash experiment, CO inhibited cytochrome c oxidase and dioxygen containing buffer are mixed, exactly as they would be in a stopped-flow experiment. In the dark, the dissociation rate of CO is very slow (Boelens et al., 1982). As a result, the reactants can be mixed well before the reaction proceeds to any significant extent. Once the mixing is complete, cytochrome a_3-CO is photolyzed with an intense flash of light, and the reaction with dioxygen begins within a microsecond.

Flash flow experiments have revealed that the reoxidation of cytochrome a and Cu_A is multiphasic. Cytochrome a_3 is reoxidized at a rate of $3 \times 10^4\ s^{-1}$. Cu_B may be reoxidized simultaneously. However, since this center lacks an easily observable spectroscopic signature, it is difficult to ascertain

whether this is the case. It appears that 40% of cytochrome a is reoxidized simultaneously with cytochrome a_3. Following this initial phase, 60% of Cu_A is reoxidized at a rate of 7×10^3 s^{-1}, and finally the balance of cytochrome a and Cu_A are reoxidized at 700 s^{-1} (Hill and Greenwood, 1984ab). These data have been interpreted in terms of a branching mechanism for the reoxidation of the enzyme in which there are two different subpopulations of the enzyme, and in each population, cytochrome a and Cu_A donate electrons to the dioxygen binding site at different rates. According to this scheme, 40% of the enzyme molecules follow a pathway in which cytochrome a is reoxidized first, and 60% follow a pathway in which Cu_A is reoxidized first (Hill and Greenwood, 1984ab; Hill et al., 1986).

Another central aspect of the intramolecular electron transfer in cytochrome c oxidase is the rate of electron equilibration between cytochrome a and Cu_A. If both cytochrome a and Cu_A are to participate in a single pathway, the rate of electron transfer between these two sites must be fast enough to support the turnover rate of the enzyme. If, on the other hand, both cytochrome a and Cu_A are capable of donating electrons directly to the dioxygen binding site, a fast cytochrome a to Cu_A electron transfer rate could short-circuit an electron gating mechanism related to proton pumping (Blair et al., 1985). In addition, there is always the possibility that the cytochrome a and Cu_A electron transfer rate could be one of the rates controlled by the enzyme as part of its gating mechanism. Toward addressing this problem, Morgan et al. (1989) studied the electron equilibration between cytochrome a and Cu_A in a CO-inhibited, three-electron-reduced form of the enzyme. They reported a value of 17,000 s^{-1} for the sum of the forward and reverse rate constants (cytochrome $a^{2+}/Cu_A^{2+} \rightleftharpoons$ cytochrome a^{3+}/Cu_A^{1+}). Thus the

electron equilibration is far faster than the turnover rate of the enzyme. The rate constant for electron transfer from Cu_A to cytochrome a was measured to be 20,000 s^{-1} in fully oxidized cytochrome c oxidase using pulse radiolysis (Kobayashi et al., 1989) and flash photolysis (Nilsson, 1992).

PROTON PUMPING

It is now generally agreed that cytochrome c oxidase is a redox-linked proton pump. Widespread attentions have recently been focused on the attempts to understand the mechanistic principle and the molecular mechanism of the enzyme's proton pumping process. Several intriguing, albeit at times speculative mechanisms have been proposed (Wikström and Krab, 1979; Babcock et al., 1983; Malmström, 1985; Gelles et al., 1986; Mitchell, 1987).

In cytochrome c oxidase, exergonic electron transfer from ferrocytochrome c to dioxygen binding site is a "downhill" process, whereas the "vectorial" proton translocation is an "uphill" process. From thermodynamic consideration, unfavorable translocation of a proton against an electrochemical gradient must be driven by the exergonic electron transfer reaction. Two different types of coupling between electron transfer and vectorial proton translocation have been suggested: direct coupling and indirect coupling. Direct coupling means that the redox center is also a proton translocator. Coupling can also be indirect, in which the redox centers may only be in conformational contact with the proton translocating elements (Wikström and Krab, 1978).

Chan and co-workers have used the concept of electron and proton "gating" to describe coupling between the flow of electrons and protons through the transducer (Blair et al., 1986; Gelles et al., 1986). Generally speaking, "gating" refers to a control of accessibility of sites. "Electron gating" means that the enzyme ensures that electron transfer occurs only when it is in an "electron input state," i.e. ready to accept electrons; and out of an "electron output state," i.e. completing the linked proton translocation, and ready to transfer electron to its acceptor. As a result, the highly exergonic electron transfer drives the uphill proton translocation process in an efficient way to attain a maximum "electron gating," meaning that electron enters the input state, followed by protonation of the proton translocation group, leads to transition of the enzyme's conformation.

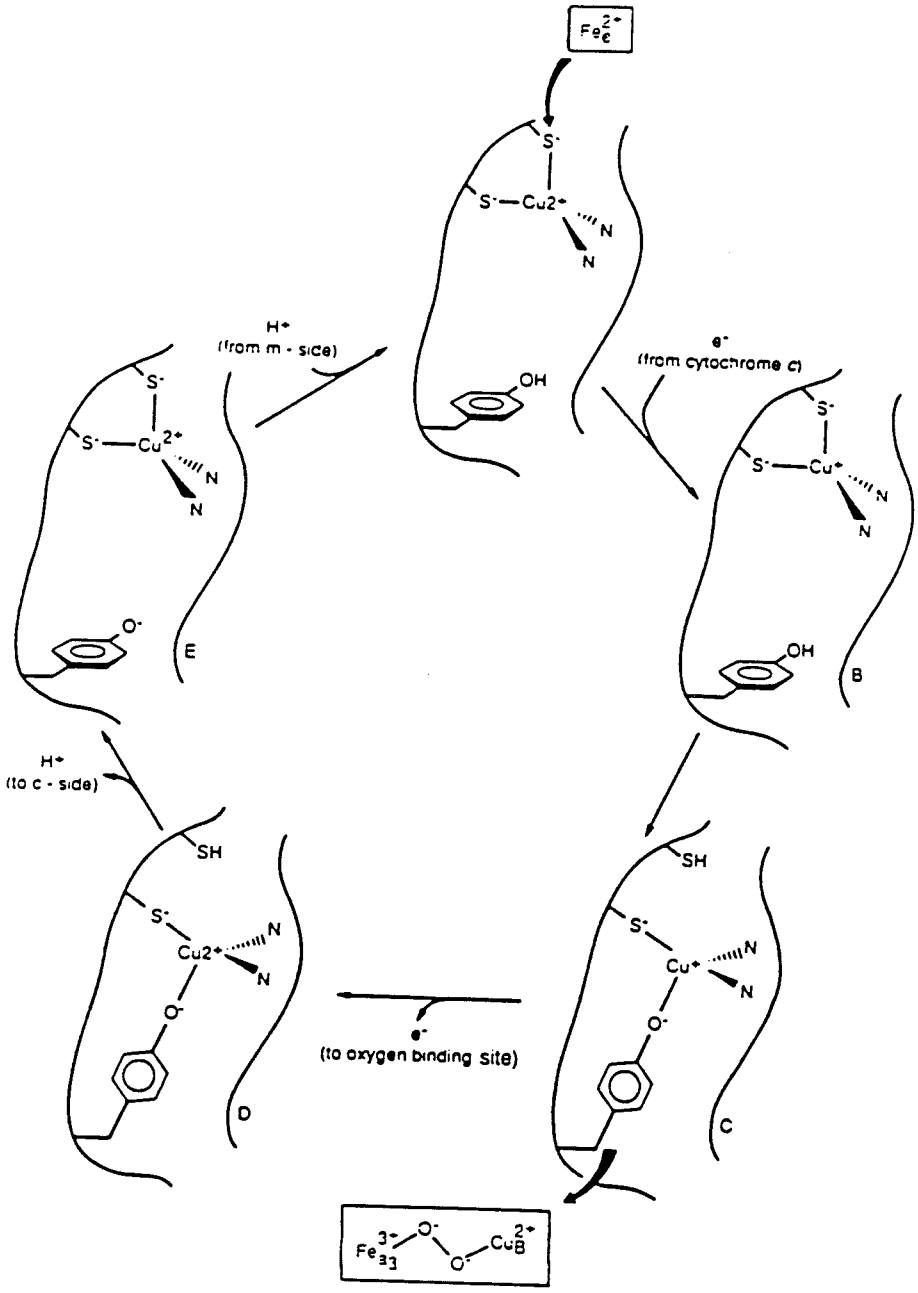
On the basis of proton pumping mechanism principles and experimental evidence which suggested the site of redox linkage, a structurally detailed model, the Chan model, has been proposed based on Cu_A as the site of redox coupling (Gelles et al., 1986). The Chan model describes the nature of proton pumping by using the concept of electron gating in addition to the redox linkage and proton gating. In the model, Chan and co-workers argued that electron gating should be an essential element of any model of a redox-linked proton pump (Gelles et al., 1986). The enzyme must be able to tune the rate constants of relevant electron transfer processes to enhance the coupled process and suppress the uncoupled pathway. To achieve the electron gating, a switch between two conformational states which correspond to the redox states of Cu_A, has been proposed. The electron enters the Cu_A site in one conformation (the "input state"), and facile electron transfer out of the site occurs only after the enzyme has switched

conformation to the "output state." The redox linkage actually occurs during this switching process. Furthermore, Chan and co-workers pointed out that the global conformation changes accompany only in the proton translocation steps during the proton pumping cycle (Gelles et al., 1986). Figure I-6 shows a detail picture of the Chan model.

Although there is no direct experimental evidence to support the Cu_A based redox-linkage model, a number of Cu_A modification experiments do suggest a central role for Cu_A in proton pumping. For example, the p-Hydroxymercuribenzoate (pHMB) modified enzyme, in which Cu_A is converted into a type 2 copper center, exhibits a rapid extravesicular alkalinization when this modified enzyme is reconstituted into membrane vesicles (Gelles and Chan, 1985; Nilsson et al., 1988). The enzyme after heat-treatment, in which Cu_A is a mixture of type 1 and type 2 copper, displays either no proton pumping activity (Sone and Nicholls, 1984) or a proton conduction behavior similar to that of the pHMB modified enzyme (Li et al., 1988). Also this model is consistent with the data available on the ligand structure of Cu_A (Li et al., 1987; Steffens et al., 1987; Hall et al., 1988).

The work described in this thesis attempts to address some open questions regarding the structure and function of cytochrome c oxidase. In Chapter II, a detailed study of zinc subunit location and function is described. Chapter III describes an electron input study from ruthenium complex modified cytochrome c to cytochrome c oxidase. Intramolecular electron transfer is also examined, and presented in Chapter IV. The final experimental chapter, Chapter V, deals with probing the protein matrix for conformational changes using fluorescence measurements.

Figure I-6: Chan model for the redox-linked proton pumping. (From Chan and Li, 1990).


Fe_a^{2+}
H+
(from n - side)
e-
(from cytochrome c)
H+
(to c - side)
e-
(to oxygen binding site)
E
B
D
C
Fe_{a3}^{3+}
Cu_B^{2+}

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II The Nature of Zinc in Cytochrome c Oxidase

Abstract.

The zinc ion in bovine heart cytochrome c oxidase can be completely depleted from the enzyme with mercuric chloride without denaturing the protein. The metal atom stoichiometry of 5Cu/4Fe/0Zn/2Mg obtained for the enzyme following $HgCl_2$ treatment indicates that this depletion is highly selective. Zinc depletion exposes one cysteine on subunit VIa and one cysteine on subunit VIb for N-iodoacetyl-N'-(5-sulfo-1-naphthyl)ethylene-diamine (1,5-I-AEDANS) labeling, suggesting that the zinc plays a structural role in the protein by providing a bridge between these two subunits. Although the treatment of cytochrome c oxidase with mercuric chloride inhibits the steady-state activity of the enzyme, subsequent removal of the Hg^{2+} bound to cysteine residues by 1,5-I-AEDANS significantly reverses the inhibition. This latter result indicates that the removal of the zinc itself does not alter steady-state activity of the enzyme. In addition, the effect of zinc depletion on the transient electron transfer activity of the protein is investigated using the laser flash photolysis of 5-deazariboflavin technique developed by Hazzard et al. (*Biochemistry*, **30**, 213, 1991). Results obtained demonstrated that the depletion of the zinc does not affect the initial electron input to the enzyme.

Introduction.

Cytochrome c oxidase is the terminal oxidase in the mitochondrial respiratory chain. It catalyzes the reduction of molecular oxygen to water by ferrocytochrome c as well as the coupling of this exergonic reaction to the uphill vectorial translocation of protons across the inner membrane of the mitochondrion. Each functional unit of the enzyme is known to contain two heme A prosthetic groups (heme a and heme a_3) and two copper ions (Cu_A and Cu_B) (Wikström et al., 1981) and up to 13 subunits (Downer et al., 1976; Kadenbach et al., 1983). It is generally accepted that heme a, heme a_3 and Cu_B reside in subunit I (Winter et al., 1980; Ludwig et al., 1980), and Cu_A is associated with subunit II (Martin et al., 1988; Hall et al., 1988). In addition, it is now established that one zinc ion and one magnesium ion as well as additional copper (Cu_x) are intrinsic components of the enzyme. A stoichiometry of 5Cu/4Fe/2Zn/2Mg has been proposed for the dimeric protein (Einarsdottir and Caughey, 1985; Pan et al., 1991a).

The zinc atom seems to be tightly bound to the enzyme. This metal ion cannot be removed by dialysis against buffers containing the chelating agents 1,10-phenanthroline or ethylenediaminetetraacetic acid (EDTA) in the pH range 6.0 to 9.5. On the other hand, Moubarak et al. (1987) showed that 60% of the zinc could be removed from the enzyme following treatment of the enzyme with fluorescein mercuric acetate (FMA). Naqui et al. (1988) also reported that zinc could be partially (up to 50%) depleted either by treating the enzyme with dipicolinic acid or by trypsin digestion. Efforts to remove this metal ion completely from the enzyme without denaturing the protein have been unsuccessful.

There have been speculations on the possible role of zinc in the structure and function of cytochrome c oxidase. Einarsdottir and Caughey (1984) have proposed that the zinc is involved in the proton pumping activity of the enzyme. Naqui et al. (1988) have suggested that the zinc ion may play a structural role. Since the subunit location of the zinc in the enzyme is still not known, these ideas cannot be tested directly. However, possible candidates for the zinc binding site have been suggested, including subunits VIa , VII, and VIIIc (Vb, VIb and VIIa, respectively in the nomenclature of Kadenbach)*, which contain 4, 4, and 2 cysteine residues, respectively (Buse et al., 1985). Yewey and Caughey (1987) have found that subunit III, subunit VII, polypeptide a, and polypeptide b (III, VIIa, Vb and VIc, respectively, in the nomenclature of Kadenbach) do not contain zinc as well as other metal ions. On the basis of extended x-ray absorption fine structure (EXAFS) measurements, Naqui et al. (1988) suggested that the zinc might reside in subunit VIa (Vb in the nomenclature of Kadenbach) coordinated to two sulfur ligands. However, in a different EXAFS experiment, Scott (1989) obtained evidence for three or four sulfur ligands and one nitrogen ligand to the zinc.

In the present study, we have completely removed the zinc from cytochrome c oxidase with mercuric chloride without affecting the other metal ions in the protein. A metal atom stoichiometry of 5Cu/4Fe/0Zn/2Mg is obtained for the zinc-depleted enzyme. This experiment represents the first successful attempt to deplete the zinc totally from the oxidase. Zinc depletion appears not to inhibit the steady-state electron transfer activity and transient electron input of the enzyme. Finally, the removal of the zinc exposes

cysteines on both subunit VIa and VIb for fluorescent labeling by N-iodoacetyl-N'-(5-sulfo-1-naphthyl)ethylene-diamine (1,5-I-AEDANS). This observation implicates these cysteines on subunit VIa and VIb as the location of the zinc ion. We propose that the zinc plays a structural role in the protein by providing a bridge between these two subunits.

Experimental Procedures.

Materials Beef heart cytochrome c oxidase was isolated by the method of Hartzell and Beinert (1974). Special precaution was taken to avoid contamination of the preparation from exogenous metal ions during the isolation and purification. Enzyme concentrations were determined spectrophotometrically by using $\Delta\varepsilon$ (reduced minus oxidized = 24 mM^{-1} cm^{-1}) at 605 nm. The enzyme preparation was stored at -80 °C until used. Tris, sodium deoxycholate, and lauryl maltoside were obtained from Sigma and used without further purification. FMA, dipicolinic acid and 1,5-I-AEDANS were also obtained from Sigma. 5-deazariboflavin was a kind gift from Prof. Tollin at University of Arizona.

Depletion of Zinc Using Mercuric Chloride A 50 μM solution of cytochrome c oxidase in 100 mM sodium phosphate, pH 7.4, containing 0.3% Tween 80 was treated with 200 μM $HgCl_2$. The incubation was carried out for 10 minutes at 37 °C. The $HgCl_2$-treated sample was then passed through a 1.5 x 20 cm Sephadex G-25 column equilibrated with 100 mM sodium phosphate, pH 7.4 containing 0.1% sodium cholate, and 10 mM EDTA to remove the zinc and excess $HgCl_2$.

Depletion of Zinc Using Dipicolinic Acid Cytochrome c oxidase (100 μM) in 100 mM sodium phosphate, 1 mM EDTA, 1% Tween 20, 9% glycerol, pH 6.0 was treated with 40 mM dipicolinic acid at 4 °C for 50 min as described by Naqui et al. (1988). After incubation, the sample was passed through a Sephadex G-25 column that was previously equilibrated with the same buffer at 4 °C.

Depletion of Zinc Using FMA Cytochrome c oxidase (50 μM) in 100 mM sodium phosphate, 0.3% Tween 80, pH 7.4 was incubated with 200 μM FMA at 37 °C for 5 min as described by Moubarak et al. (1987). The solution was then passed through a Sephadex G-25 column that had previously been equilibrated with 100 mM sodium phosphate, 0.1 % sodium cholate, 10 mM EDTA, pH 7.4.

Dissociation of Cysteine-Bound Hg^{2+} from the $HgCl_2$-Treated Enzyme by 2-Mercaptoethanol Sodium deoxycholate was added to a $HgCl_2$-treated oxidase sample (20 μM) to a final concentration of 1%. The sample then was incubated with 120 mM 2-mercaptoethanol at 27 °C for 20 min. The excess 2-mercaptoethanol was removed by passing the sample through a Sephadex G-25 column that had previously been equilibrated with 100 mM Tris-Cl, 0.1% lauryl maltoside, pH 8.0.

Cytochrome c Oxidase Labeling with 1,5-I-AEDANS Native and four zinc-depleted oxidase preparations were labeled by 1,5-I-AEDANS as described by Hall et al. (1988). Before the labeling, all five samples (native; $HgCl_2$-treated; $HgCl_2$ + 2-mercaptoethanol-treated; FMA-treated; and dipicolinic acid-treated) were changed into a buffer of 100 mM Tris-Cl, 0.1% lauryl

maltoside, pH 8.0. The samples were then concentrated to 50 μM, reduced anaerobically with 4 mM dithiothreitol, and 5 mM 1,5-I-AEDANS was added. The labeling reaction was carried out for 12 h at 4 °C in the dark. Finally, the solution was chromatographed on a 1 x 20 cm Sephadex G-25 column equilibrated with 50 mM sodium phosphate, 0.1% lauryl maltoside, pH 7.4.

Assay of Electron Transfer Activity The activities of the native and zinc-depleted enzymes were determined spectrophotometrically using a Beckman DU-7400 diode array spectrophotometer. Assays were performed by following the oxidation of 20 μM ferrocytochrome c at 550 nm in 50 mM sodium phosphate, 0.1 % lauryl maltoside, pH 6.0. The concentration of cytochrome c oxidase was 5-20 nM. The turnover number of the native enzyme was typically about 500 s^{-1}.

Transient Absorption Kinetics Before the laser photolysis study, the zinc-depleted enzyme was changed into a buffer of 10 mM Tris-Cl, 0.1% lauryl maltoside, pH 7.4. The sample was then concentrated to about 400 μM by Amicon concentrator 30. Transient absorption measurements were carried out by laser flash photolysis of the reaction solution as described by Hazzard et al. (1991). The reaction solution contained 20 μM bovine cytochrome c, 10-50 μM zinc-depleted enzyme, 0.5 mM EDTA, 0.1% lauryl maltoside, 100 mM NaCl and 100 μM 5-deazariboflavin in Tris buffer at 25 °C. The oxidation of ferrocytochrome c was monitored at 550 nm and the reduction of heme a in oxidase was detected at 605 nm. The absorption transients of both 550 nm and 605 nm were fitted to two exponential first-order decay with apparent rate constant k_{obs} by using a weighted linear least-squares program.

Metal Analysis Metal contents of the enzyme preparations were determined by direct current plasma atomic emission spectrometry (DCP-AES) as described by Pan et al. (1991a). These experiments were performed in the Laboratory of Dr. Stolper, Division of Geological and Planetary Science at Caltech.

SDS-Polyacrylamide Gel Electrophoresis Cytochrome c oxidase samples were dissociated into subunits for 1 h at 25 °C in 8M urea, 5% SDS. Slab gels were run on a LKB 2001 vertical electrophoresis unit as described by Darley-Usmar et al. (1981) using a 7% polyacrylamide stacking gel and a 14% running gel, both containing 6M urea. The gels were illuminated with UV light and photographed to observe the AEDANS and FMA fluorescence. Finally, the gels were stained with Coomassie Blue.

Results.

Metal Contents of Native and Zinc-Depleted Cytochrome c Oxidase The average metal atom ratios of native and three zinc-depleted samples are presented in Table I. Three determinations were made for each sample. The experimental error of an individual measurement is within ±5%. The stoichiometry of 5Cu/4Fe/2Zn/2Mg per dimer obtained for the native enzyme, is consistent with the determination of Yoshikawa et al. (1988) and the data recently reported from our laboratory (Pan et al., 1991a). The metal contents of the dipicolinic acid-treated and FMA-treated samples confirm that about 43% and 51% of the zinc are depleted by these treatments, respectively, as determined earlier (Moubarak et al., 1987; Naqui et al., 1988). On the other hand, a metal stoichiometry of 5Cu/4Fe/0Zn/2Mg was obtained for the

Table I: Metal Contents of Zinc-Depleted Cytochrome c Oxidase

	Metal Atom ratios[a]			Assignment (per dimer)						
Method of Treatment	**Cu/Fe**	**Zn/Fe**	**Mg/Fe**	**Fe_a**	**Fe_{a3}**	**Cu_A**	**Cu_B**	**Cu_X**	**Zn**	**Mg**
Native Enzyme	1.27 ± 0.04	0.47 ± 0.02	0.55 ± 0.05	2	2	2	2	1	2	2
+ 1,5-I-AEDANS Labeling	1.23 ± 0.03	0.44 ± 0.02	0.56 ± 0.04	2	2	2	2	1	2	2
Dipiolinic Acid Treatment	1.25 ± 0.02	0.27 ± 0.02	0.60 ± 0.04	2	2	2	2	1	1	2
+ 1,5-I-AEDANS Labeling	1.24 ± 0.06	0.27 ± 0.03	0.59 ± 0.01	2	2	2	2	1	1	2
FMA Treatment	1.28 ± 0.05	0.23 ± 0.04	0.59 ± 0.01	2	2	2	2	1	1	2
+ 1,5-I-AEDANS Labeling	1.26 ± 0.02	~ 0.08	0.54 ± 0.06	2	2	2	2	1	0	2
$HgCl_2$ Treatment	1.21 ± 0.02	~ 0.01	0.60 ± 0.03	2	2	2	2	1	0	2
+ 1,5-I-AEDANS Labeling	1.24 ± 0.05	~ 0.01	0.61 ± 0.02	2	2	2	2	1	0	2

[a] **Error reported as standard deviation of three analyses.**

$HgCl_2$-treated sample. Thus, it is possible to completely deplete the zinc from the enzyme without affecting the other metal centers.

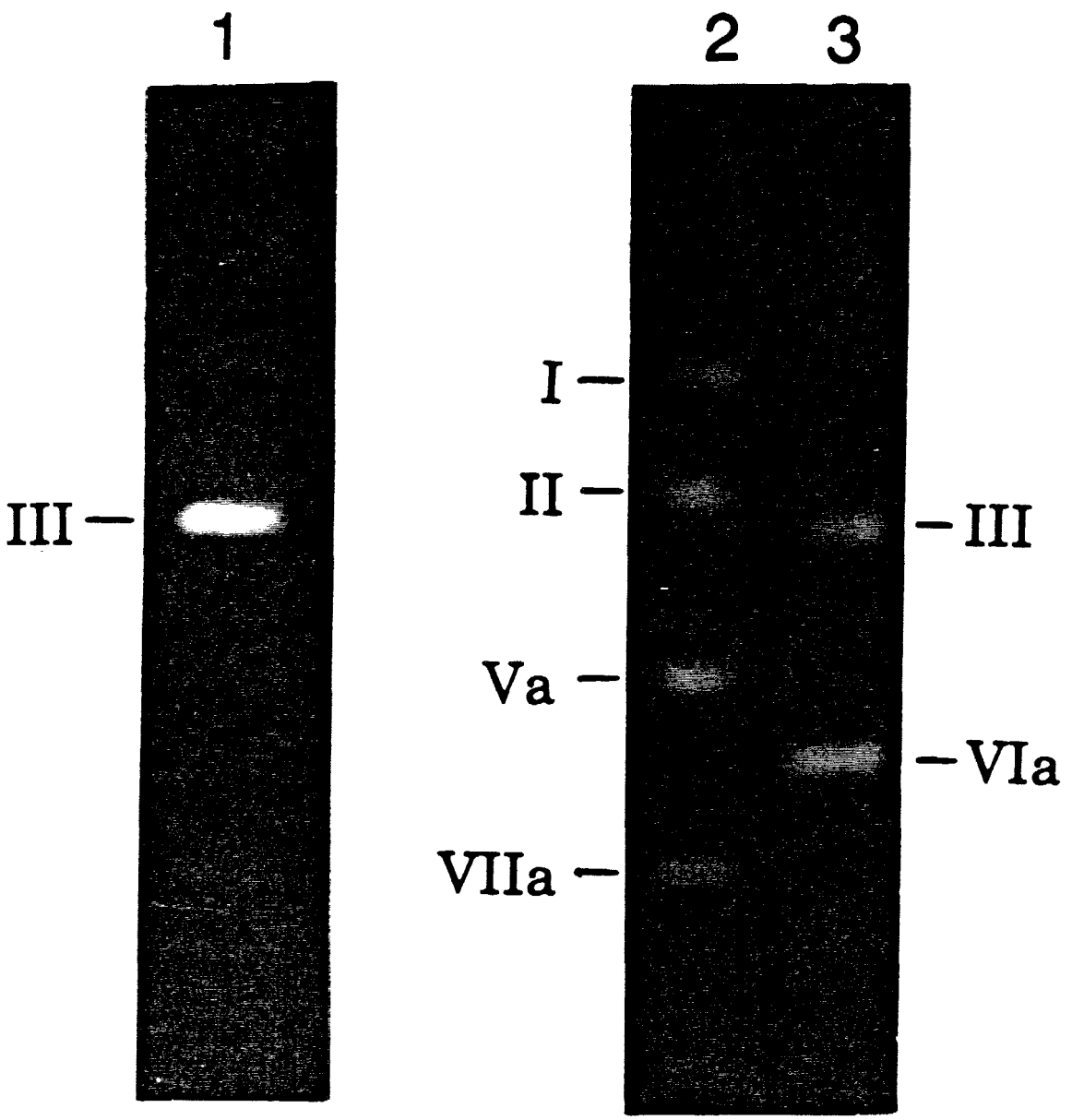
Metal Contents of 1,5-I-AEDANS Labeled-Cytochrome c Oxidase The metal contents of 1,5-I-AEDANS labeled-cytochrome c oxidase samples are also summarized in Table I. From these results, we see that the metal contents of native, $HgCl_2$-treated and dipicolinic acid-treated samples remain unchanged after the labeling. For the FMA-treated enzyme, however, we find that the residual zinc is also removed after 1,5-I-AEDANS-labeling. As shown in Table I, the Zn/Fe ratio decreases from 0.23 to 0.08, indicating that the remaining 50% of the total zinc is removed by 1,5-I-AEDANS labeling. A metal stoichiometry of 5Cu/4Fe/0Zn/2Mg was obtained for the FMA + 1,5-I-AEDANS-labeled sample.

Labeling of Cytochrome c Oxidase by 1,5-I-AEDANS 1,5-I-AEDANS labels free cysteines in the enzyme. Since cysteines have been implicated as ligands to the zinc ion, removal of the zinc might possibly expose these cysteine residues for labeling by 1,5-I-AEDANS. As shown in lane 1 of Figure II-1, 1,5-I-AEDANS exclusively labels subunit III in the native enzyme, presumably one of the exposed cysteines (Hall et al., 1988). In the case of the dipicolinic acid-treated sample, subunit VIa and/or VIb (subunit VIa and VIb cannot be discerned) are labeled in addition to subunit III (lane 3 of Figure II-1). This result suggests exposure of a cysteine(s) on the subunits upon removal of 43% of the zinc from the enzyme by dipicolinic acid treatment.

SDS-polyacrylamide gel electrophoresis of the FMA-treated enzyme reveals FMA fluorescence from subunits I, II, Va and VIIa (lane 2 of Figure II-

Figure II-1. SDS-Polyacrylamide Gel Electrophoresis of FMA- and 1,5-I-AEDANS-Labeled Cytochrome c Oxidase

50 μM native cytochrome c oxidase (*lane 1*) and dipicolinic acid-treated enzyme (*lane 3*) were treated with 4 mM dithiothreitol and 5 mM 1,5-I-AEDANS for 12 h in 100 mM Tris-Cl, 0.1% lauryl maltoside, pH 8.0, and passed through a Sephadex G-25 column to remove excess 1,5-I-AEDANS. *Lane 2*, 50 μM native cytochrome c oxidase in 100 mM sodium phosphate, 0.3% Tween 80, pH 7.4 was incubated with 200 μM FMA at 37 °C and passed through a sephadex G-25 column to remove excess FMA. 15 μL aliquots of each sample were then dissociated for 1 h at 25 °C in 5% SDS and 8M urea and run on a 14% polyacrylamide Laemmli gel system. The slab gel was photographed with UV illumination to detect the fluorescence and then stained with Coomassie Blue to locate the subunits as indicated.


1
2
3
III
I
II
III
Va
VIa
VIIa

1), consistent with the earlier results of Stonehuerner et al. (1985). Unfortunately, these results cannot be used to infer the cysteine(s) that have been modified *in situ* by the FMA treatment since it is well-known that the FMA mercurial reagent migrates during the denaturing conditions of the gel. However, if the FMA treatment is followed by covalent labeling by 1,5-I-AEDANS prior to gel electrophoresis to dissociate the subunits, the cysteine(s) exposed by the zinc depletion may be inferred. The results of such a "double" labeling experiment show extensive FMA or 1,5-I-AEDANS labeling of subunits I, II, III, Va, VIa, VIb and VIIa, as judged by the FMA and/or 1,5-I-AEDANS fluorescence (data not shown). It is clear from this experiment that cysteines of subunits VIa and VIb have become labeled by 1,5-I-AEDANS upon the total depletion of the zinc.

A similar 1,5-I-AEDANS-labeling pattern is obtained for the $HgCl_2$-treated sample (lane 2 of Figure II-2; Figure II-3), where we observe extensive labeling of subunits I, II, III, Va, VIa, VIb and VIIa. It is clear that the $HgCl_2$ treatment loosens up the protein and exposes a number of cysteine residues for 1,5-I-AEDANS labeling. However, if the $HgCl_2$-treated oxidase were incubated with 2-mercaptoethanol to dissociate the bound Hg^{2+} prior to 1,5-I-AEDANS labeling, we observe labeling of only subunits II, III, VIa and VIb (lane 1 of Figure II-2; Figure II-3). 1,5-I-AEDANS labels subunit III even in the native enzyme. The cysteine(s) modified on subunit II have previously been identified as cysteines 196 and/or 200, which become exposed upon removal of Cu_A by 2-mercaptoethanol (Hall et al., 1988). By elimination then, we can conclude that total zinc depletion by the $HgCl_2$-treatment exposes cysteines on subunits VIa and VIb to 1,5-I-ADEANS labeling. We obtain a 1:1 fluorescence intensity ratio for the 1,5-I-AEDANS-labeled subunits VIa and VIb. This

Figure II-2. SDS-Polyacrylamide Gel Electrophoresis of Cytochrome c Oxidase Labeled with 1,5-I-AEDANS.

Lane 1, $HgCl_2$ + 2-mercaptoethanol treated cytochrome c oxidase; *lane 2*, $HgCl_2$-treated cytochrome c oxidase; *lane 3*, native enzyme. All samples were labeled by 1,5-I-ADEANS as described in the text and the legend for Figure 1. The subunits were dissociated for 1 h at 25 °C in 5% SDS and 8 M urea and run on Laemmli gel system as described in the text. The slab gels were photographed with UV illumination to observe the fluorescence.

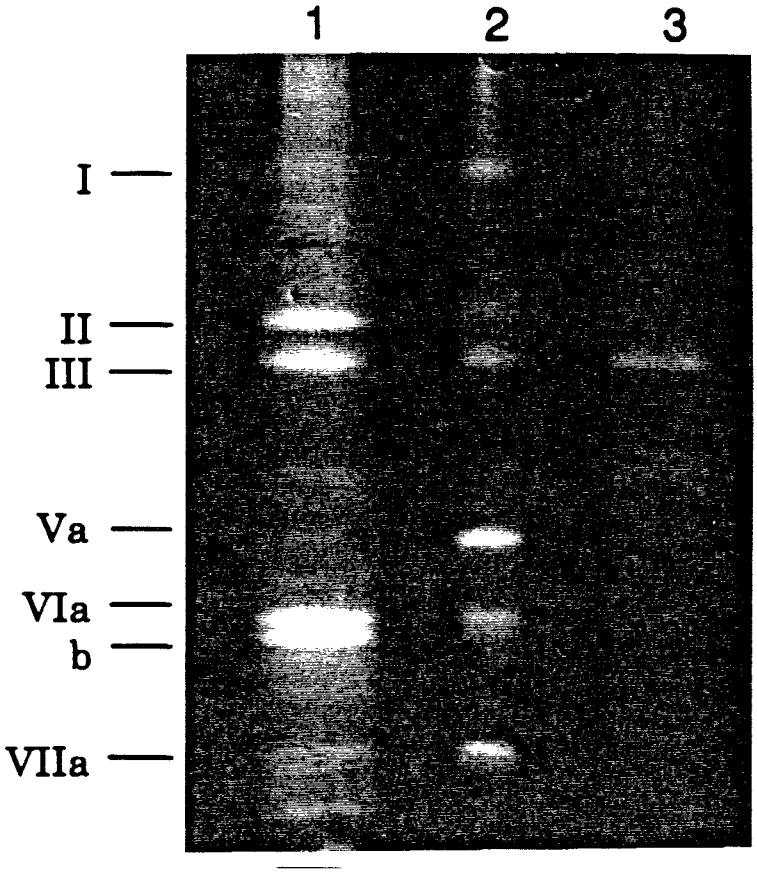
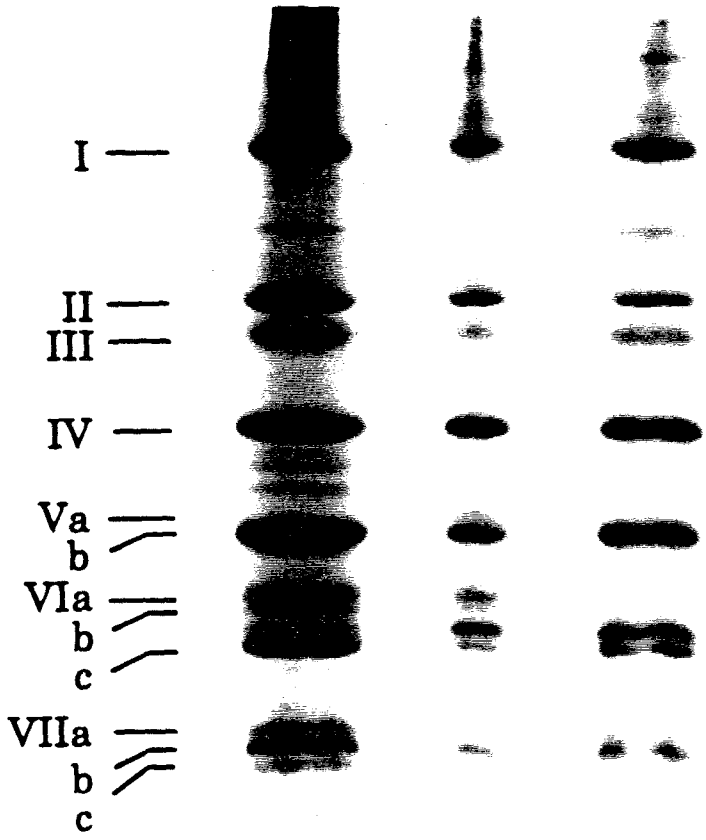

1
2
3
I
II
III
Va
VIa
b
VIIa

Figure II-3. SDS-Polyacrylamide Gel Electrophoresis of Cytochrome c Oxidase Native and various modified cytochrome c oxidase samples were incubated with 5% SDS and 8 M urea for 1 h at 25 °C. Afterwards, these samples were run on Laemmli gel system as described in the text. The slab gels were stained by Coomassie Blue to locate the subunits as indicated.


I
II
III
IV
Va
b
VIa
b
c
VIIa
b
c

result implicates cysteine residues on subunits VIa and VIb as ligands to the zinc ion in the native enzyme.

Electron Transfer Activity of the Zinc-Depleted Oxidase The steady-state electron transfer activities of the various zinc-depleted cytochrome c oxidases are compared with that of the native oxidase in Table II. Labeling of the native oxidase with 1,5-I-AEDANS does not alter the steady-state electron transfer activity. The electron transfer activity of the dipicolinic acid-treated sample is also essentially the same as that of the native enzyme. Both the $HgCl_2$-treated and FMA-treated samples display a 50% decrease in the electron transfer activity, but the inhibitions are reversed significantly after subsequent incubation of the samples with 1,5-I-AEDANS. This result indicates that the inhibition of the activity is caused by the binding of the mercuric compounds rather than the result of zinc depletion.

Figure II-4 shows the transient of the intracomplex electron transfer between cytochrome c and the zinc-depleted cytochrome c oxidase. The reduction of ferrocytochrome c by 5-deazariboflavin semiquinone and its subsequent reoxidation by oxidase was monitored at 550 nm (Figure II-4a). The kinetic trace is biphasic and fitted well to a sum of two exponential first-order decays with apparent rate constant k_{obs}. A rate constant of 950 s^{-1} is obtained for the fast phase and 80 s^{-1} for the slow phase. The reduction of heme a in the zinc-depleted enzyme was detected at 605 nm (Figure II-4b). The kinetic trace is also biphasic and fitted well with a fast rate of 1050 s^{-1} and a slow rate of 90 s^{-1}. Here, there is an excellent match between the reoxidation of ferrocytochrome c and the reduction of heme a in both the fast and slow phases. Also a 1:1 molar ratio of ferrocytochrome c reoxidized to heme a

Tabel II: Steady-State Electron Transfer Activities of Zinc-Depleted Cytochrome c Oxidase

Treatment	Activity (%)
Native Enzyme	100
+ 1,5-I-AEDANS Labeling	95
Dipicolinic Acid Treatment	90
+ 1,5-I-AEDANS Labeling	80
FMA Treatment	40
+ 1,5-I-AEDANS Labeling	65
$HgCl_2$ Treatment	45
+ 1,5-I-AEDANS Labeling	75

Figure II-4a. Intracomplex electron transfer between cytochrome c and zinc-depleted cytochrome c oxidase. 20 μM bovine cytochrome c and 20 μM zinc-depleted cytochrome c oxidase were added to 5 mM Tris buffer at pH 7.4 containing 1 mM EDTA, 0.1% lauryl maltoside, 100 μM 5-deazariboflavin, and 100 mM KCl. The sample cuvette was degassed and subjected to a N_2 dye laser (BBQ at 390 nm) flash. The reduction and the subsequent reoxidization of cytochrome c was followed at 550 nm.

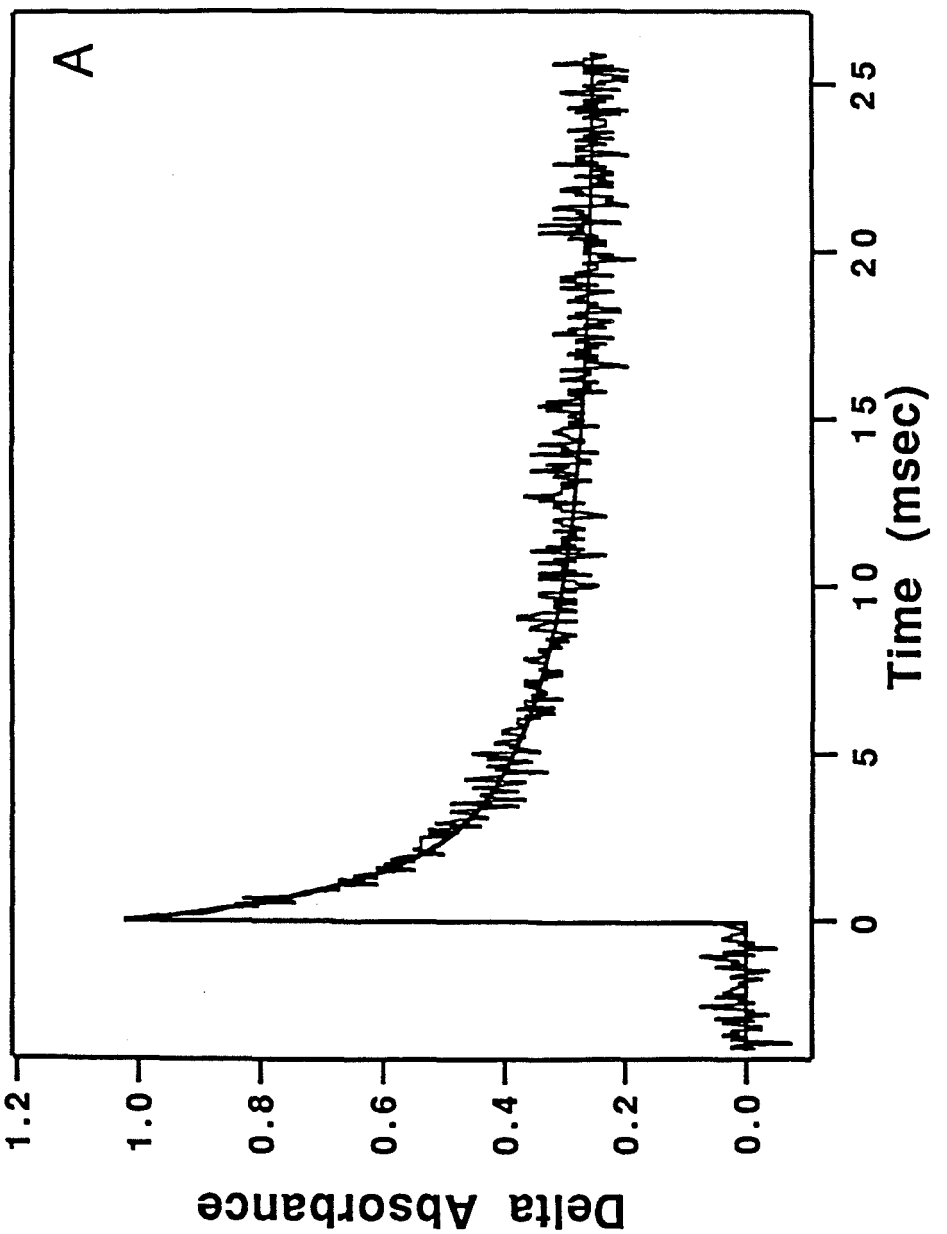
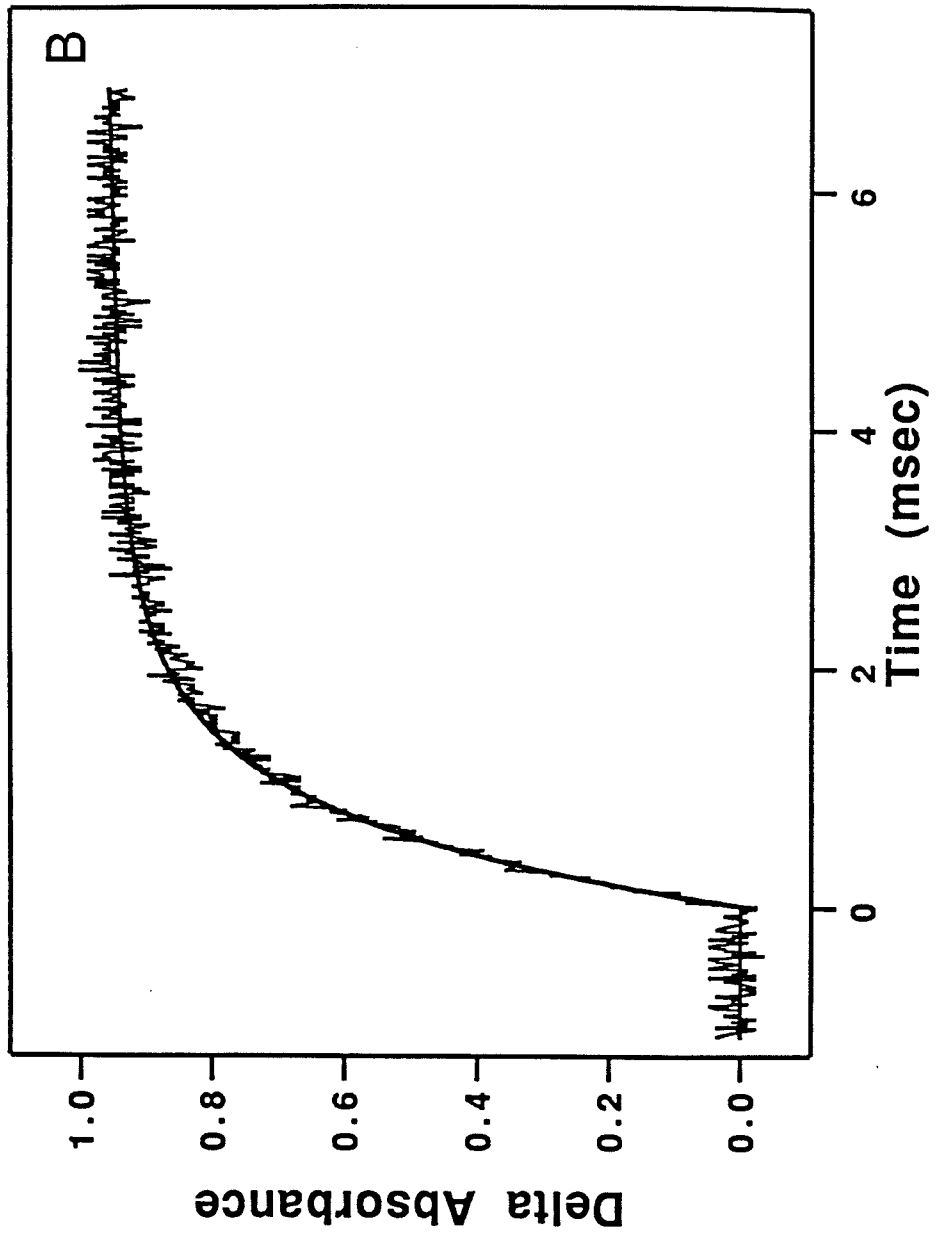

A
Delta Absorbance
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
Time (msec)

Figure II-4b. Intracomplex electron transfer between cytochrome c and zinc-depleted cytochrome c oxidase. The experimental condition and procedure are described in Figure 4a. The reduction of cytochrome a in zinc-depleted cytochrome c oxidase is monitored at 605 nm.


B
Delta Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
Time (msec)

reduced was obtained for the electron input reaction of the zinc-depleted enzyme, indicating that the electron transferred from ferrocytochrome c remains at the heme a site prior to the subsequent electron transfer to the binuclear center.

The Electron Transfer Kinetics and Transient Intracomplex Formation

For the zinc-depleted sample, the enzyme concentration dependence of the intracomplex electron transfer was also investigated (Figure II-5). It was found that the rate constant for the fast kinetic phase (k_{obs}) has a hyperbolic dependence, suggesting that initial electron input proceeds via a mechanism in which a 1:1 transient complex is formed between ferrocytochrome c (Cyt c) and cytochrome c oxidase (CcO). The complex formation and pertinent rate constants are summarized as follows:

$$\text{Cyt } c^{2+} + (CcO)_{ox} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Cyt } c^{2+}{:}(CcO)_{ox} \qquad (1)$$

$$\text{Cyt } c^{2+}{:}(CcO)_{ox} \rightarrow \text{Cyt } c^{3+} + (CcO)_{red.} \qquad (2)$$

Using the steady-state approximation, where $(CcO)_{ox} >> \text{Cyt } c^{2+}$, we obtain:

$$k_{obs} = \frac{k_1 k_{et} (CcO)_{ox}}{k_1(CcO)_{ox} + k_2 + k_{et}}. \qquad (3)$$

Under conditions where saturation occurs, k_{et} becomes rate limiting, i.e.:

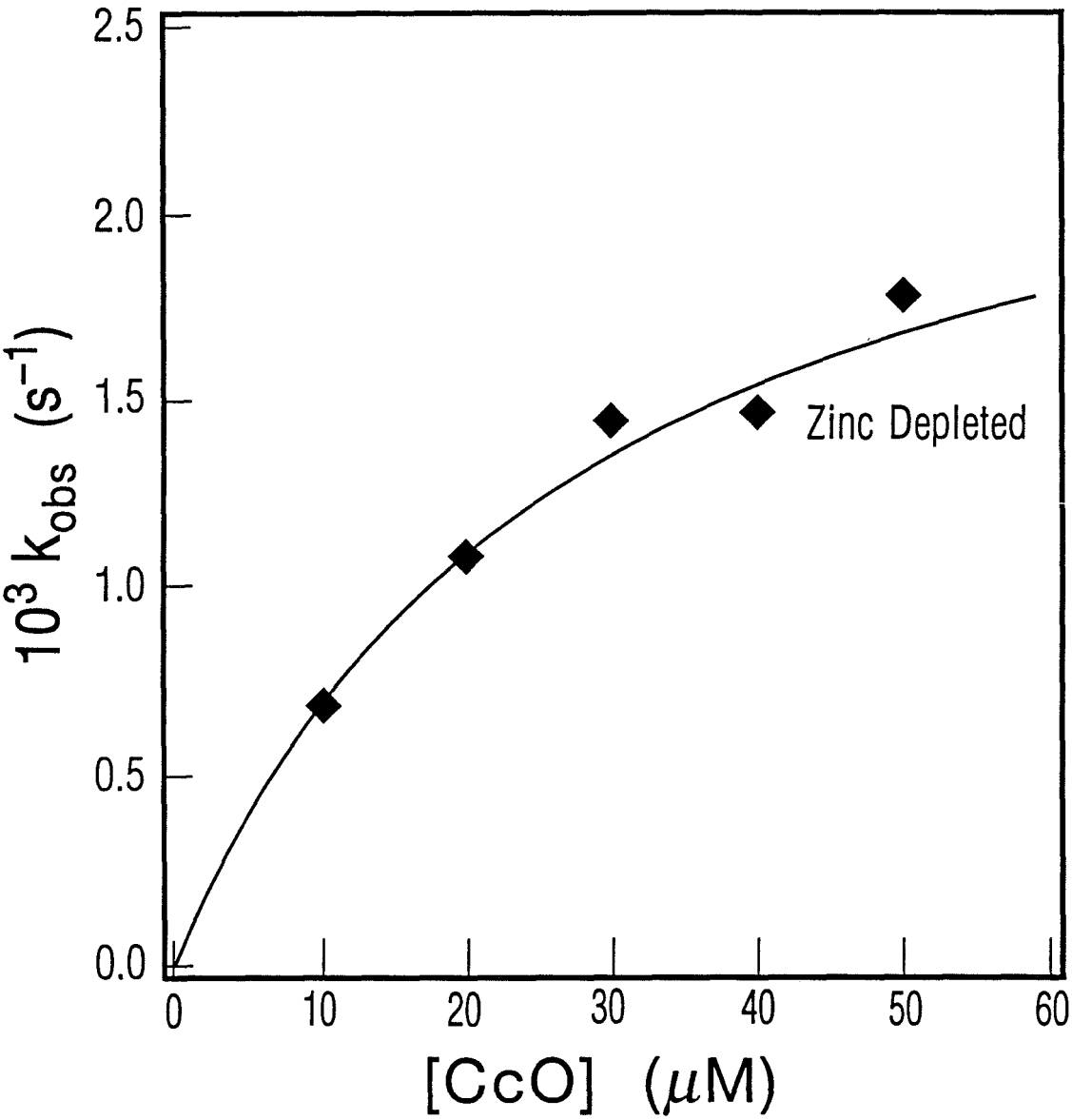
$$k_{et} << k_1(CcO)_{ox} + k_2$$

then the equation (3) is simplified to:

$$k_{obs} = \frac{k_{et} K_A (CcO)_{ox}}{K_A(CcO)_{ox} + 1'} \qquad (4)$$

where $K_A = k_1 / k_2$ is the association constant for the formation of the ferrocytochrome c:cytochrome c oxidase.

Figure II-5. Kinetics of electron transfer from ferrocytochrome c to zinc-depleted oxidase at ionic strength of 110 mM. The reaction conditions were the same as described in Figure 4a, except for addition of the zinc-depleted sample to increase its concentration. The pseudo first-order rate constants for the reduction of cytochrome a during the fast phase are plotted as a function of the concentration of zinc-depleted enzyme. The solid curves represent the best fits of the data to equation (4) in the text.


2.5
2.0
1.5
1.0
0.5
0.0
$10^3 K_{obs}$ (s^{-1})
Zinc Depleted
0
10
20
30
40
50
60
[CcO] (μM)

The best fit to the data shown in Figure II-5 yields K_A and k_{et} for the zinc depleted enzyme, which is listed in Table III. Compared to the data of native enzyme reported previously by Pan et al. (1991b), almost the same K_A was obtained for this modified enzyme, indicating that the modification does not affect the transient intracomplex formation. The rate of intracomplex electron transfer of the zinc-depleted enzyme is also similar to that of the native enzyme (2580 s^{-1}) (Pan et al., 1991b). The observed electron transfer rate of the slow phase at different oxidase concentrations has been found unchanged for the zinc-depleted enzyme in our experiment.

Discussion.

The nature of the zinc in cytochrome c oxidase, including its subunit location and the role of the zinc in the structure and function of the enzyme, has attracted considerable attention in recent years. The ideal approach for ascertaining the role of the zinc is to prepare a zinc-depleted oxidase. However, a number of laboratories have reported that it is very difficult to remove the zinc completely from the enzyme; and typically, the zinc is released only when the protein is denatured. The experiment we report here represents the first successful attempt to totally deplete the zinc without denaturing the enzyme. $HgCl_2$-treated samples give a metal stoichiometry of 5Cu/4Fe/0Zn/2Mg, indicating that the method that we have developed here to deplete the zinc is highly selective. Although both FMA and $HgCl_2$ are nonpolar mercuric compounds, FMA can remove only up to 51% of the zinc. This result suggests that the binding domain of the zinc may be relatively inaccessible. This explanation can also account for why dipicolinic acid can deplete only 43% of the zinc from the protein. It is also noteworthy that

Table III: Kinetic Parameters of Intracomplex Electron Transfer Between Cytochrome c and Cytochrome c Oxidase

Sample	E (mV)[a]	K_A (10^{-4} M^{-1})	k_{et} (s^{-1})
Native	280	5.4 ± 0.5[b]	2580 ± 30[b]
Zinc Depleted	280	3.8 ± 0.8	2650 ± 40

[a] From the work of Li et al. (1991). E is the intrinsic potential of Cu_A when cytochrome a_3 and Cu_B are prereduced, and the potential is referred to NHE.

[b] From the work of Pan et al. (1991b).

incubation of the FMA-treated enzyme with 1,5-I-AEDANS removed the remaining zinc. This protocol provides an alternate approach to prepare the zinc-depleted oxidase.

The steady-state electron transfer activities of the zinc-depleted samples indicate that the zinc does not have a profound influence on the enzymatic activity. Although partial inhibition of the enzyme is observed after $HgCl_2$ and FMA treatments, this inhibition can be largely reversed by subsequent 1,5-I-AEDANS labeling, in accordance with the earlier observations of Mann and Auer (Mann and Auer, 1980). The partial inactivation of the enzyme by mercuric compounds has been attributed to the binding of mercuric compound to a cysteine in subunit I that is crucial for electron transfer (Mann and Auer, 1980; Stonehuerner et al., 1985). Since Hg^{2+} ions bound to the cysteines can be removed by thiol-exchange, incubation of $HgCl_2$- or FMA-treated oxidase with 1,5-I-AEDANS should reverse the inactivation, as observed. In contrast to the inhibition of the steady-state activity noted here, the pre-steady-state reduction of cytochrome c oxidase by ferrocytochrome c is not affected by FMA treatment (Moubarak et al., 1987). It will be of interest to compare the pre-steady-state kinetics and proton pumping activities of the zinc-depleted oxidase with these same properties of the native enzyme.

The zinc-depleted enzyme exhibits transient electron transfer kinetic characteristics similar to those of the native enzyme (Pan et al., 1991b). This result indicates that zinc depletion does not affect the initial electron input process. Also this result confirms that zinc does not involve in the electron transfer activity of cytochrome c oxidase. It is interesting to note that about the same K_A values were obtained for the zinc-depleted enzyme and the

native enzyme, suggesting that the dissociation of the subunit VIa and VIb as a result of zinc removal does not alter the binding of the oxidase enzyme with cytochrome c.

It is very interesting that both subunits VIa and VIb are labeled after the zinc is removed (and after any substituted Hg^{2+} had been displaced by 1,5-I-AEDANS). This result directly implicates these two subunits as the zinc binding domain. On the other hand, Yewey and Caughey (1987) found that removal of subunits III, Vb, VIa, and VIIa did not affect the zinc content in the enzyme. Since the removal of subunit VIb was not confirmed in this experiment, there is a possibility that the zinc remained associated with the subunit VIb after the subunit VIa removal. If the subunit VIb was also removed by the treatment (Penttila, 1983), then our present findings would be in contradiction to the earlier results of Yewey and Caughey. However, subunits Vb, VIb, and VIIa have also been suggested as the possible subunit location of the zinc (Buse et al., 1985). On the basis of EXAFS studies, Naqui et al. (1988) and Scott (1989) have indicated that the zinc is ligated by two or three cysteines. Subunits VIa alone can provide for only one of the cysteines of the zinc, one of the other cysteine rich subunit must be involved as well. The results of the present study are consistent with a ligand structure of the zinc wherein the zinc is liganded to one cysteine on subunit VIa and one cysteine on subunit VIb. If the two cysteines are derived from two subunits, namely VIa and VIb, then we may conclude that the zinc plays a structural role in the enzyme by providing a bridge between these two subunits. In the dipicolinic acid-treated sample, 1,5-I-AEDANS labeling occurs on subunit VIa and/or VIb (subunit VIa and VIb cannot be discerned), providing further evidence in support of the subunit location of the zinc.

In conclusion, we show in this work that: 1) The zinc ion of cytochrome c oxidase can be completely dissociated from the enzyme without denaturing the protein; 2) The zinc does not appear to be involved in the electron transfer activity of the enzyme; and 3) The zinc serves a structural role in the enzyme by providing a bridge between subunit VIa and VIb.

*The subunit nomenclature of Kadenbach (Kedenbach et al., 1983) is used throughout this Chapter.

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III The Binding and Electron Transfer of Ru(bipyridine)2(dicarboxybipyridine)(lysine-86) Ferricytochrome c to Cytochrome c Oxidase

Abstract.

A cytochrome c derivative labeled at lysine 86 with ruthenium bis(bipyridine) dicarboxybipyridine [$Ru^{II}(bpy)_2(dcbpy)$] (Ru-86-cytochrome c) was prepared as previously described by Pan et al. [Pan, L.P.; Durham, B.; Wolinska, J.; and Millett, F. (1988) *Biochemistry* 27, 7180-7184]. Transient absorption measurements did not show evidence associated with intramolecular electron transfer from the Ru^{II*} to the heme group (k_1). It is estimated that both the rate constants of electron transfer from the Ru^{II*} to Fe^{III} in cytochrome c (k_1) and the thermal recombination from the ferrous heme to the Ru^{III} (k_2) are slow, less than 1×10^5 s^{-1}. Laser excitation (in the presence of aniline) of the complex formed between Ru-86-cytochrome c and bovine cytochrome c oxidase at low ionic strength resulted in rapid electron transfer within the ruthenated cytochrome c derivative, followed by electron transfer to the cytochrome c oxidase. The observable intracomplex rate constants for the oxidation of cytochrome c are 560 s^{-1} for the fast phase and 114 s^{-1} for the slow one. The rate constant for the reduction of cytochrome a in cytochrome c oxidase is 2.3×10^4 s^{-1}. However, there is a fast kinetic phase at 550 nm which is beyond the resolution capability of the instrument. No direct electron transfer to Cu_A was observed at 830 nm. Apparently, the bulky ruthenated moiety near the binding domain between cytochrome c and cytochrome c oxidase alters the binding orientation of the cytochrome c *vis* a *vis* the cytochrome c oxidase at the docking site, so that cytochrome c preferentially transfers its electron directly to cytochrome a rather than to Cu_A. The intracomplex electron transfer rate exhibited the expected ionic strength dependence.

Introduction.

Cytochrome c oxidase (CcO) is an integral membrane protein which catalyzes the oxidation of ferrocytochrome c and the corresponding reduction of dioxygen to water. The catalytic function of CcO is carried out using four redox active metal centers. One pair of these centers, cytochrome a_3 and Cu_B, forms a binuclear cluster, where dioxygen is bound and reduced during the catalytic cycle. The other, cytochrome a and Cu_A, mediates the flow of electrons from ferrocytochrome c to the binuclear center (Wikström et al., 1981; Chan and Li, 1990). The electrons enter the protein from the cytosol side of the mitochondrial inner membrane, and the protons consumed in the dioxygen chemistry are taken up from the matrix. In this manner, the sideness of the membrane is exploited to convert redox free energy into a proton electrochemical gradient across the inner mitochondrial membrane. In addition, concurrent with this electron flow, protons can be vectorially pumped across the inner membrane from the matrix to the cytosol to augment the transmembrane electrochemical gradient.

It is generally assumed that the elucidation of the electron transfer pathway(s) between the four redox centers in CcO may provide relevant information concerning the mechanism of redox-linked proton translocation in CcO. Toward this end, it is essential to determine how CcO accepts electrons from cytochrome c. A number of approaches have been developed to study the electron transfer reaction between cytochrome c and CcO. Initially, the stopped-flow technique was used to investigate the electron transfer reaction between ferrocytochrome c and CcO (Wilson et al., 1975; Antalis and Palmer, 1982). Since the stopped-flow method is limited by the

time course of mixing of the two proteins and the binding of cytochrome c to CcO, more recent studies have focused on rapid photo-initiated electron transfer and transient absorption experiments in preformed complexes of the redox protein partners. Cusanovich and Tollin developed a technique which utilizes flavins to photochemically initiate electron transfer in redox complexes (Cusanovich and Tollin, 1980). Hazzard et al. specifically used this technique to measure the intracomplex electron transfer reaction between cytochrome c and CcO (Hazzard et al., 1991). In a different approach, Hill developed the flow-flash technique to follow the electron transfer within a complex of cytochrome c and reduced CO-inhibited cytochrome c oxidase in the presence of dioxygen (Hill, 1991).

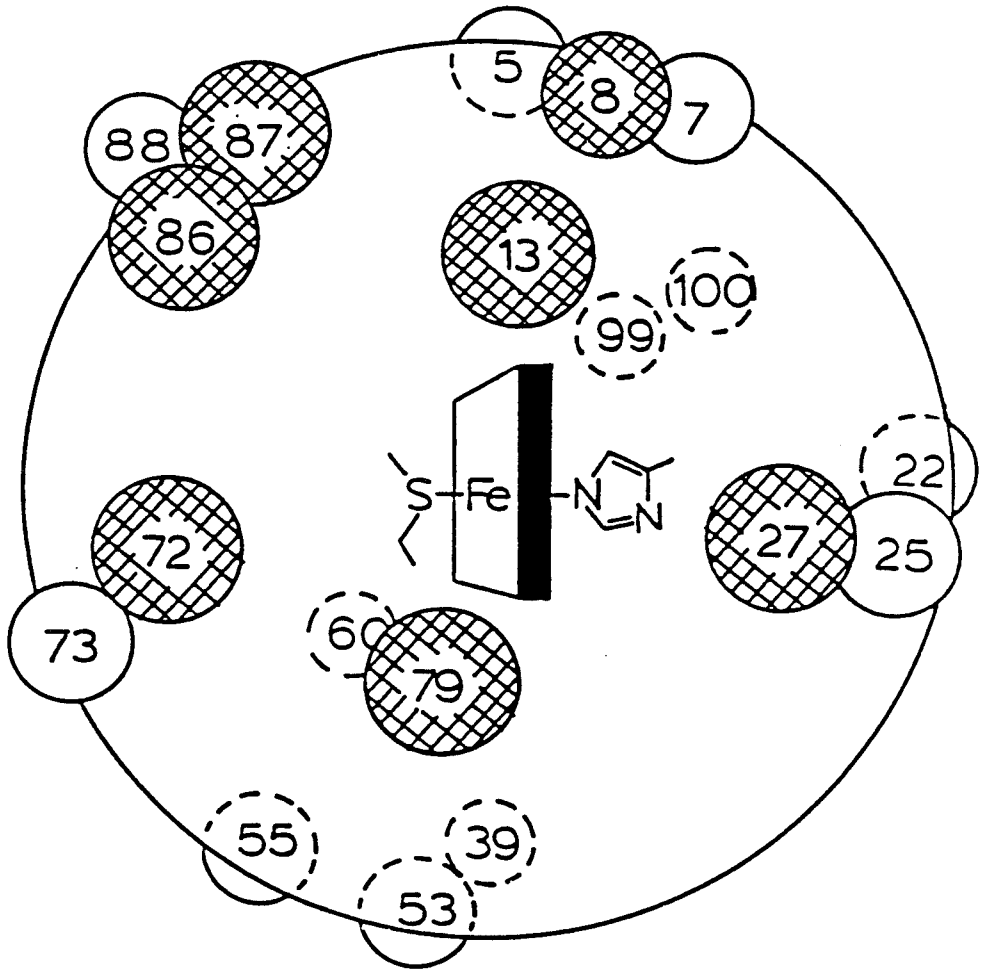
In yet a different strategy, Winkler et al. (1982) and Isied et al. (1982) introduced the use of tethered ruthenium complexes $[Ru^{II}(NH_3)_5]$ to specifically coordinate to histidine 33 of cytochrome c. Along similar lines, Millett and coworkers modified cytochrome c covalently with a tris(bipyridine)ruthenium(II) complex at various lysine amino groups (Pan et al., 1988). In these ruthenated cytochrome c's, the photoexcited Ru^{II} group can transfer an electron to the heme in cytochrome c on a nanosecond time scale (Durham et al., 1989). All these groups exploited the photophysics of the ruthenium(II) to inject an electron into the iron heme, which can subsequently be transferred to CcO. A series of these derivatives have been used to study the electron transfer reaction between cytochrome c and CcO (Pan et al., 1993). As an example the photo-reduced heme in the cytochrome c derivative modified at lysine 25, which is on the periphery of the heme crevice domain, can transfer an electron to Cu_A with a rate constant of $1.1 \times 10^4\ s^{-1}$. In derivatives modified at lysines which are remote from the

cytochrome c heme, such as lysines 7, 39, 55 and 60, the electron transfer from the cytochrome c to Cu_A can occur at a rate greater than 10^5 s^{-1}. In a subsequent step the electron is transferred from Cu_A to cytochrome a within the oxidase with a rate constant of 2×10^4 s^{-1} (Pan et al., 1993). In contrast, for cytochrome c derivatives modified at lysines 13 and 27 in the heme crevice domain, the rate constants are much slower, with the intracomplex rate constants for oxidation of cytochrome c ranging from 1000 to 6000 s^{-1}; no reduction of Cu_A was observed at 830 nm.

In recent years it has been generally accepted that Cu_A is the primary electron acceptor from cytochrome c in CcO. However, at least in studies of electron transfer in transient complexes formed between cytochrome c and CcO under high ionic strengths, the data support two electron input pathways, one through Cu_A and the other cytochrome a (Pan et al., 1991). In support of this, the experiments undertaken under low ionic strengths indicate that the electron transfer pathway between cytochrome c and CcO is dependent on the details of the binding between the two redox partners in the preformed complex. To further elucidate the effects of the binding of cytochrome c to CcO on the electron transfer pathway between the two proteins, we have now studied the electron transfer between ruthenium-lysine 86 cytochrome c derivative and CcO. Lysine 86 is located at the upper-left side of the heme crevice in cytochrome c, and this lysine is believed to be involved in the binding domain to CcO (Figure III-1) (Smith et al., 1977; Smith et al., 1981; Ferguson-Miller et al., 1978; Rider and Bosshard, 1980).

Experimental Procedures.

Figure III-1: Schematic diagram of horse heart cytochrome c viewed from the front of the heme crevice. Those lysine residues involve in the binding interaction with cytochrome c oxidase are indicated by checkered circles. Other lysine residues located on the front and back side of cytochrome c are indicated by closed circles and dashed circles, respectively.

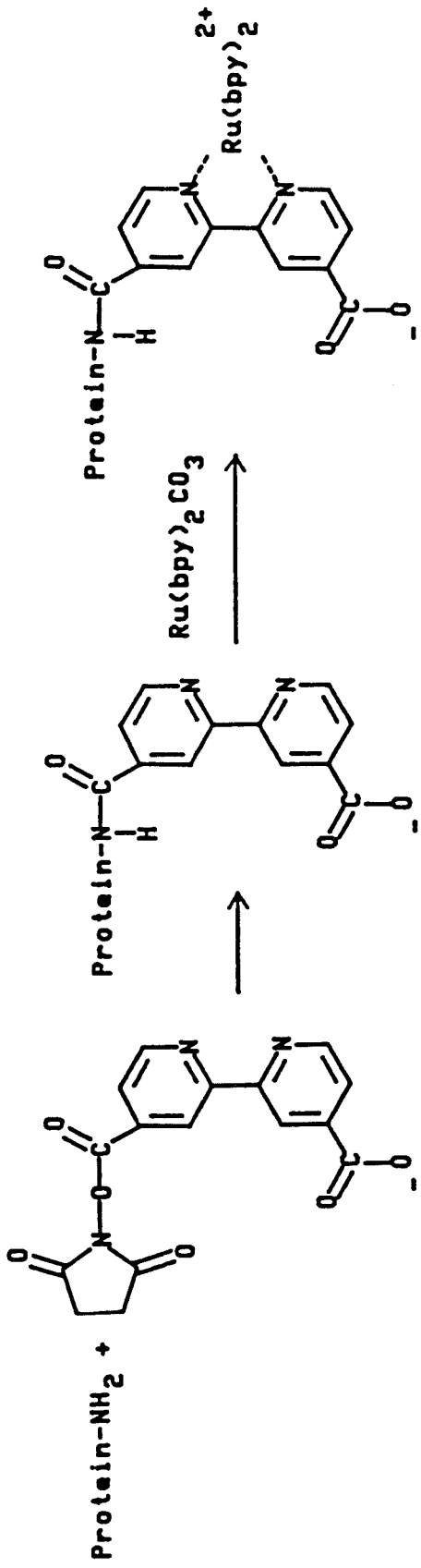

5
8
7
88
87
86
13
100
99
S
Fe
N
N
22
27
25
72
73
60
79
55
39
53

Materials. Horse heart cytochrome c (Type VI) and aniline were obtained from Sigma Chemical Co. and used without further purification. Cytochrome c oxidase was isolated and purified by the method of Hartzell and Beinert (1974), and was stored at –80°C until needed. The enzyme preparation had a turnover number of 500 s^{-1}.

Preparation of Mono-N-Hydroxysuccinimide Ester of Dicarboxybipyridine. The preparation scheme of ruthenium complex labeled cytochrome c derivative is shown in Figure III-2. Dicarboxybipyridine (dcbpy) (0.32 mm_ol in 3 mL water) was titrated slowly with 0.32 mm_ol KOH (molar ratio equals 1) to convert one of the two protonated carboxyl groups to the K^+ salt. After the sample was dried under a stream of nitrogen, 1.2 mL of dry DMF was added. The solution was stirred at 25 °C for 1 h, then 0.32 mm_ol N, N'-dicyclohexylcarbodiimide and 0.32 mm_ol N-Hydroxysuccinimide were added in a molar ratio of 1. The reaction was kept at 25 °C for 15 h as described by Anderson et al. (1964). The precipitated dicyclohexylurea was removed, and the DMF was evaporated under a stream of nitrogen. The mono-N-hydroxysuccinimide ester of dcbpy was dissolved in 2 mL of 50 mM Tris-Cl, pH 8.0. The small amount of the di-N-hydroxysuccinimide ester of dcbpy present in the original reaction mixture has a low solubility in the Tris buffer and was removed by centrifugation.

Preparation of Dcbpy-Cytochrome c Derivatives. Horse heart cytochrome c (20 mM in 2 mL of 50 mM Tris-Cl, pH 8.0) was incubated with freshly prepared mono-N-hydroxysuccinimide ester of dcbpy (80 mM in 2 mL of 50 mM Tris-Cl buffer, pH 8.0). The reaction was kept at 25 °C for 2 h. Then 7 mL water was added to stop the reaction. The sample was applied to a 2.5 x

Figure III-2: Preparation scheme of the $Ru^{II}(bpy)_2(dcbpy)$–cytochrome $\underline{c}$ derivative.


Protein-NH2 +
Protein-N
H
Ru(bpy)2CO3
Ru(bpy)2
2+

70 cm column of Bio-Rex 70. The column was eluted with an exponential gradient from 50 mM ammonium phosphate, pH 7.2, to 160 mM ammonium phosphate, pH 7.2. Seven fractions obtained were concentrated individually either on a small Bio-Rex 70 column or by an Amicon concentrator 10. Each of the concentrated fractions was rechromatographed twice on a 1 x 20 cm column of Whatman sulfopropyl SE-53; the exponential gradient used was from 20 mM to 250 mM sodium phosphaste, pH 6.0.

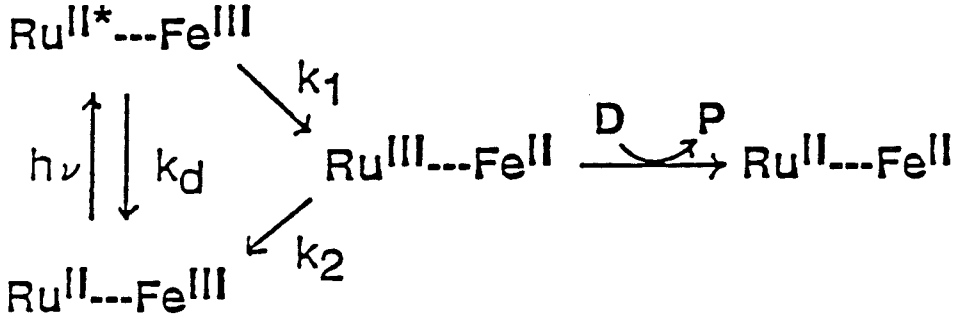
Preparation of Ru Bis(bipyridine) Dicarboxybipyridine-Cytochrome c Derivative. Each of the purified dcbpy-cytochrome c fractions was concentrated to 1 mM and incubated anaerobically with 10 mM $Ru^{II}(bpy)_2CO_3$ in 100 mM sodium acetate, pH 4.0, for 24 h at 25 °C in the dark, then a small Bio-Gel P-2 column was used to remove excess reagent. The sample was purified on a 0.6 x 45 cm Whatman CM-32 column using a gradient from 20 to 400 mM sodium phosphate, pH 6.0.

Characterization of $Ru^{II}(bpy)_2(dcbpy)$-Cytochrome c Derivative. UV-visible spectra of the derivative was recorded on a Hewlett-Packard 8452a diode array spectrophotometer. The identification of the lysine modified in the derivative was carried out by HPLC and amino acid analysis. First the derivative was exchanged into 100 mM bicine, pH 8.0 with an Amicon concentrator 10. The derivative (1 mg/mL) was then digested with 50 ng/μL TPCK-treated trypsin for 15 h at 37 °C. The tryptic digests were separated on a Dynamax 300-Å reverse-phase HPLC column using a linear gradient from 0.01% trifluoroacetic acid to 100% methanol. The eluent was monitored at 210 and 450 nm. The amino acid composition of each purified peptide was determined by hydrolyzing in 6 N HCl containing 0.1% 2-mercaptoethanol for

22 h at 110 °C in an evacuated, sealed tube. The hydrolysates were injected into a microbore amino acid analyzer equipped with ninhydrin detection (Smith et al., 1977).

Transient Absorption Measurements. Intramolecular electron transfer transient absorbance measurements were carried out by laser flash photolysis of 2 ml solutions contained in a 1 cm quartz optical cuvette. The solutions contain 20 μM derivative in 100 mM sodium phosphate, pH 7.0, at 25 °C. For the experiments on electron transfer between cytochrome c and CcO, 10 μM $Ru(bpy)_2(dcbpy)$-Lys 86 cytochrome c was mixed with 15 μM CcO in 5 mM sodium phosphate, pH 7.0, containing 0.1% dodecyl maltoside and 10 mM aniline, 1 mM 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy free radical (3CP). The aniline and 3CP were added as electron quencher to re-reduce Ru^{III} in order to prevent the internal back reaction noted earlier (Scheme I).

Photolysis was accomplished with 480 nm pulse from a Lambda Physik FL 3002 dye laser using coumarin 102 dye, which was pumped by a Lambda Physik LPX 210i excimer laser. The light pulse width is about 25-ns with an energy of about 1.5 mJ. Single wavelength transient absorption kinetics were obtained using a 75W xenon arc lamp as a probe source. The signal was passed through an Instruments SA 1680B double monochromator and detected with a photomultiplier tube. The transient signal was amplified using either a 200MHz quasi differential amplifier (for kinetics out to 750 μs) or a LeCroy DSP 1402E programmable amplifier (for kinetics out to 10 ms) and digitized using a Tektronix R710 200 MHz transient digitizer interfaced to a 386-based microcomputer.



Scheme I

The transient absorption measurements at 830 nm were conducted in the laboratory of Prof. Francis Millett at the University of Arkansas. All the transient absorption measurements carried out at other wavelengths were also repeated in Prof. Francis Millett's laboratory. The instrumental set up as well as the method of measurement have been described by Durham et al. (1989) and Geren et al. (1991).

Photo-Induced Electron Transfer. Photoinduced electron-transfer reaction from Ru^{II*} to the ferric heme group (k_1) and for the thermal recombination from the ferrous heme group to Ru^{III} (k_2) can be detected by the proper choice of wavelengths (Scheme I). The transient observed at 556.5 nm is assigned to Ru^{II*} since this is an isobestic point for cytochrome c. The transient for Ru^{II*} can also be obtained from the luminescence transient decay at 630 nm. The absorption transient of Fe^{II} is observed at 550 nm, which is the characteristic wavelength for $Fe^{II/III}$ oxidation state change. Considering that Ru^{II*} at 556.5 nm will contribute a bit to the absorption transient at 550 nm, the absorption transient of Fe^{II} is obtained by subtracting the Ru^{II*} transient at 556.5 nm from the Fe^{II} transient at 550 nm using appropriate extinction coefficients. The recovery of Ru^{II} following a laser pulse is the main contributor to the absorption transient at 440 nm. The small contribution at 440 nm due to heme Fe^{II} was taken into account and subtracted by the transient at 550 nm (Durham et al., 1989).

Intracomplex Electron Transfer. The rate of electron transfer within the cytochrome c: CcO complex was measured at a number of different wavelengths. The reduction and reoxidation of cytochrome c was monitored at 550 nm using the extinction coefficient of $\Delta\varepsilon_{550} = 18.6\ mM^{-1}cm^{-1}$

. The reduction of cytochrome a was followed at 604 nm using $\Delta\varepsilon_{604}$=16 mM^{-1} cm^{-1}. The reduction of Cu_A was detected at 830 nm using $\Delta\varepsilon_{830}$ = 2.00 mM^{-1} cm^{-1} (Blair et al., 1983). The detection system at 830 nm has been described by Pan et al. (1993). The absorbance transients were analyzed with the KINFIT kinetics program for single and double-exponential decays.

Results.

Preparation of Ru(bpy)$_2$(dcbpy)-Cytochrome c derivative.

N-hydroxysuccinimide has been widely used as a carboxyl activating group in peptide synthesis (Anderson et al., 1964), and the mono-N-hydroxysuccinimide ester of dcbpy was found to react very efficiently with the lysine amino group on horse heart cytochrome c. As shown in Figure III-3, dcbpy-cytochrome c was separated into seven fractions on the Bio-Rex 70 column. SDS gel electrophoresis demonstrated that no cross-linked dimers were formed, indicating that no di-N-hydroxysuccinimide ester of dcbpy was present in the reaction mixture. Besides the seven main fractions and unreacted cytochrome c, some multiply labeled cytochrome c were obtained. It was found that the yield of the seven main fractions could not be increased by using more mono-N-hydroxysuccinimide ester of dcbpy. After repurification on a Whatman SE-53 column, fraction 1 were resolved into subfractions 1A and 1B as shown in Figure III-4. Spectral analysis indicated that subfractions 1A and 1B contained a single equivalent of dcbpy. Each of the purified dcbpy-cytochrome c fractions was then incubated with $Ru(bpy)_2CO_3$ at pH 4.0 to form the Ru(bpy)$_2$(dcbpy)-cytochrome c derivatives. A control experiment indicated that no residues on native cytochrome c reacted with $Ru(bpy)_2CO_3$ under the experiment conditions, although

Figure III-3: Purification of $Ru(bpy)_2(dcbpy)$-cytochrome c derivatives. The crude reaction mixture of dcbpy-cytochrome c (500 mg) was chromatographed on a 2.5 x 70 cm Bio-rex 70 column using an exponential gradient from 50 mM ammonium phosphate, pH 7.2, to 160 mM ammonium phosphate, pH 7.2. The absorption was measured at 542 nm.

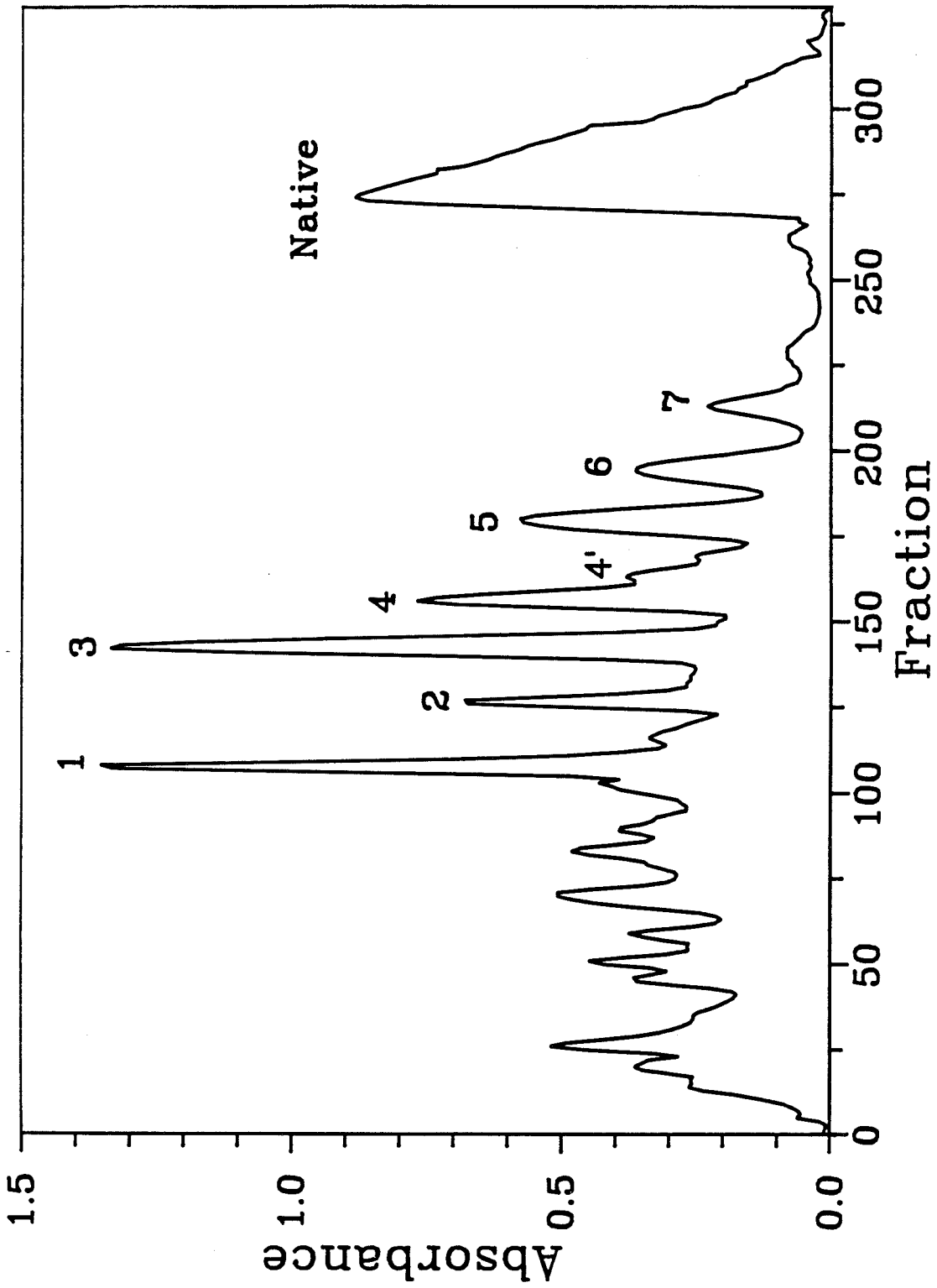
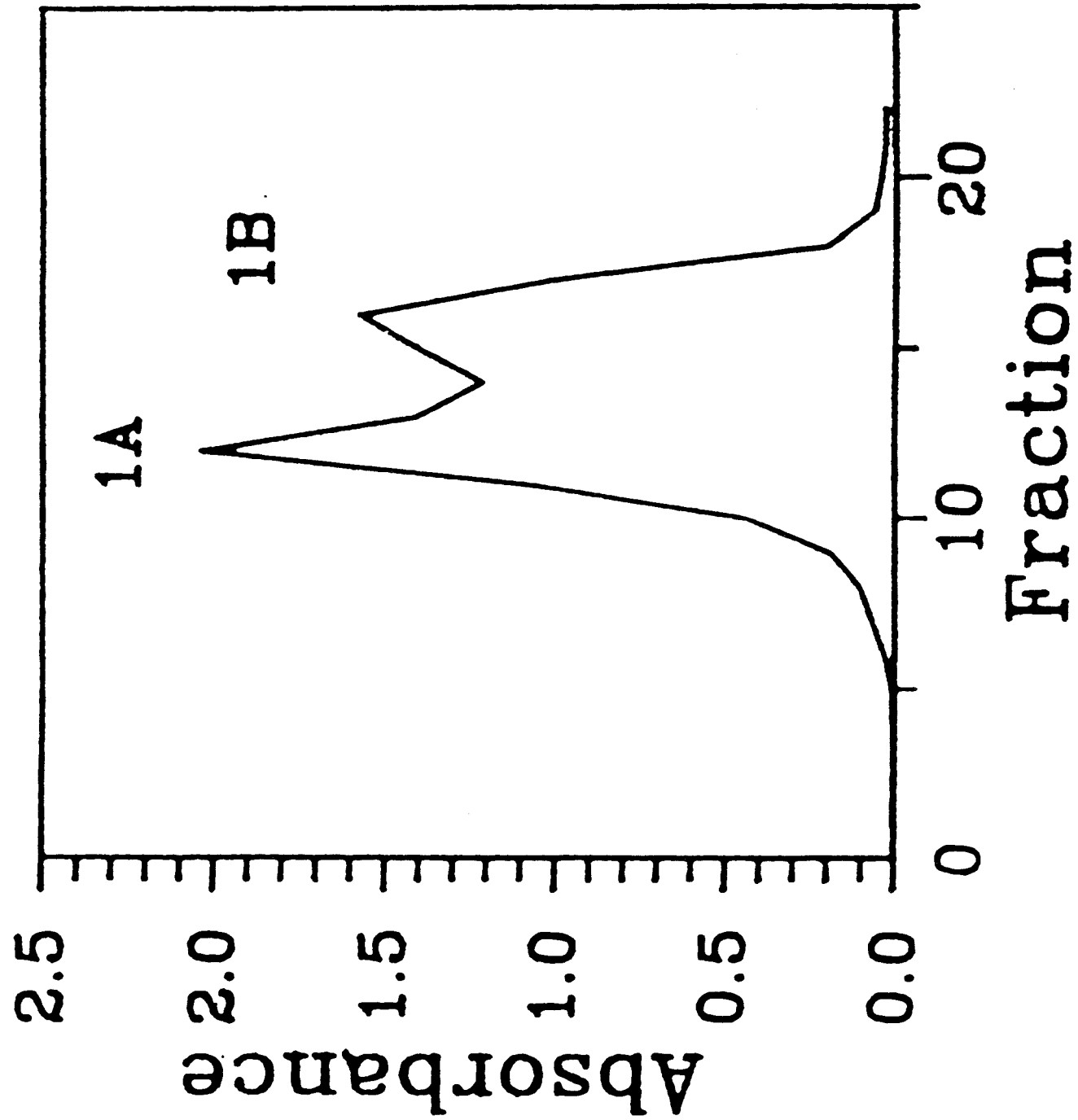

Native
1
2
3
4
4'
5
6
7
Absorbance
1.5
1.0
0.5
0.0
0
50
100
150
200
250
300
Fraction

Figure III-4: Purification of $Ru(bpy)_2(dcbpy)$-cytochrome c derivatives. Fraction 1 from Figure III-3 was rechromatographed on a 1.5 x 25 cm column of Whatman sulfopropyl SE-53 using an exponential gradient from 20 to 250 mM sodium phosphate, pH 6.0. The absorption was measured at 542 nm.


1A
1B
Absorbance
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
Fraction

histidine 33 did react at pH values above 6.0 (Durham et al., 1990). Each of the derivatives was purified on a Whatman CM-32 column. A very small band due to unmodified dcbpy-cytochrome c was resolved from a major band that contained one equivalent of $Ru(bpy)_2(dcbpy)$ per cytochrome c.

Identification of $Ru(bpy)_2(dcbpy)$ cytochrome c Derivative Modified at Lysine 86. The lysine residue modified with ruthenium complex was determined by reverse-phase HPLC of a tryptic digest and amino acid analysis. Figure III-5 shows the HPLC chromatograms of native cytochrome c and cytochrome c derivative modified with $Ru(bpy)_2(dcbpy)$ at lysine 86. The difference between the chromatograms of native and modified cytochrome c is that a new ruthenium labeled peptide appeared. Amino acid analysis confirms this derivative was singly labeled with ruthenium complex at lysine 86.

Photo-Induced Electron Transfer Kinetics. Photoexcitation of the Ru-lysine 86 derivative did not exhibit transient associated with electron transfer to heme group at 550 nm. We did see a fast transient at 550 nm, but later we found this transient was the same at 550 nm as that at 556.5 nm. So the transient at 550 nm with very fast speed was due to the excited state Ru^{II*}. However, we observed a transient absorption change at 550 nm at slow time scale (shown in Figure III-6). It seems that the rate constant k_1 for electron transfer from Ru^{II*} to Fe^{III} and the rate constant k_2 for back electron transfer from Fe^{II} to Ru^{III} were very slow. k_1 and k_2 are both less than 1×10^5 s^{-1}.

Intracomplex Electron Transfer Kinetics. Laser flash photolysis of Ru-lysine 86 cytochrome c resulted in rapid electron transfer from Ru^{II*} to the

Figure III-5: HPLC chromatogram of the tryptic digests of a. Ru-86-cytochrome c derivative b. Native cytochrome c. The tryptic digests were separated on C-18 reverse-phase HPLC using a linear gradient from 0.01% trifluoroacetic acid to 100% methanol. The eluent was monitored at 450 nm.

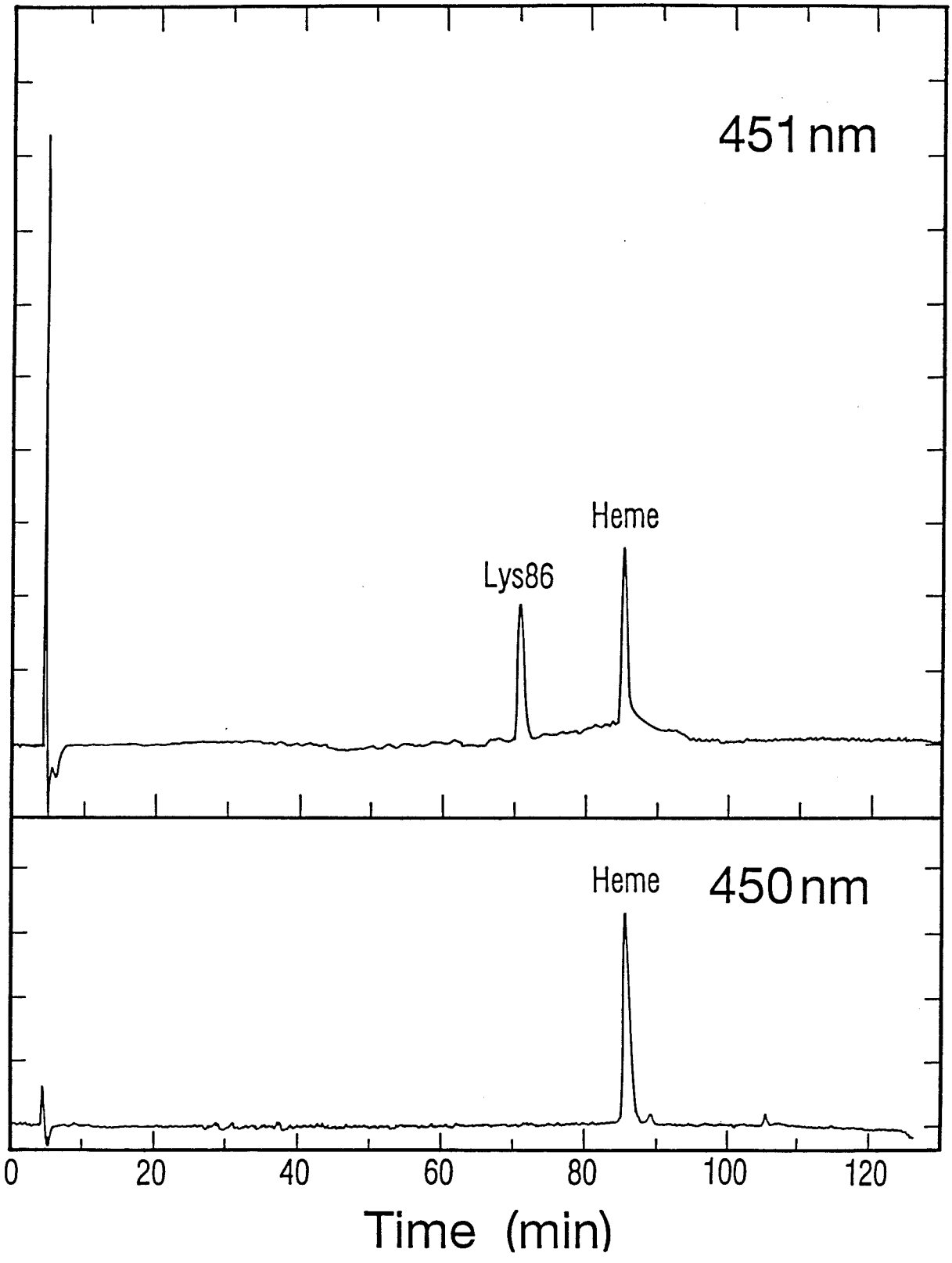
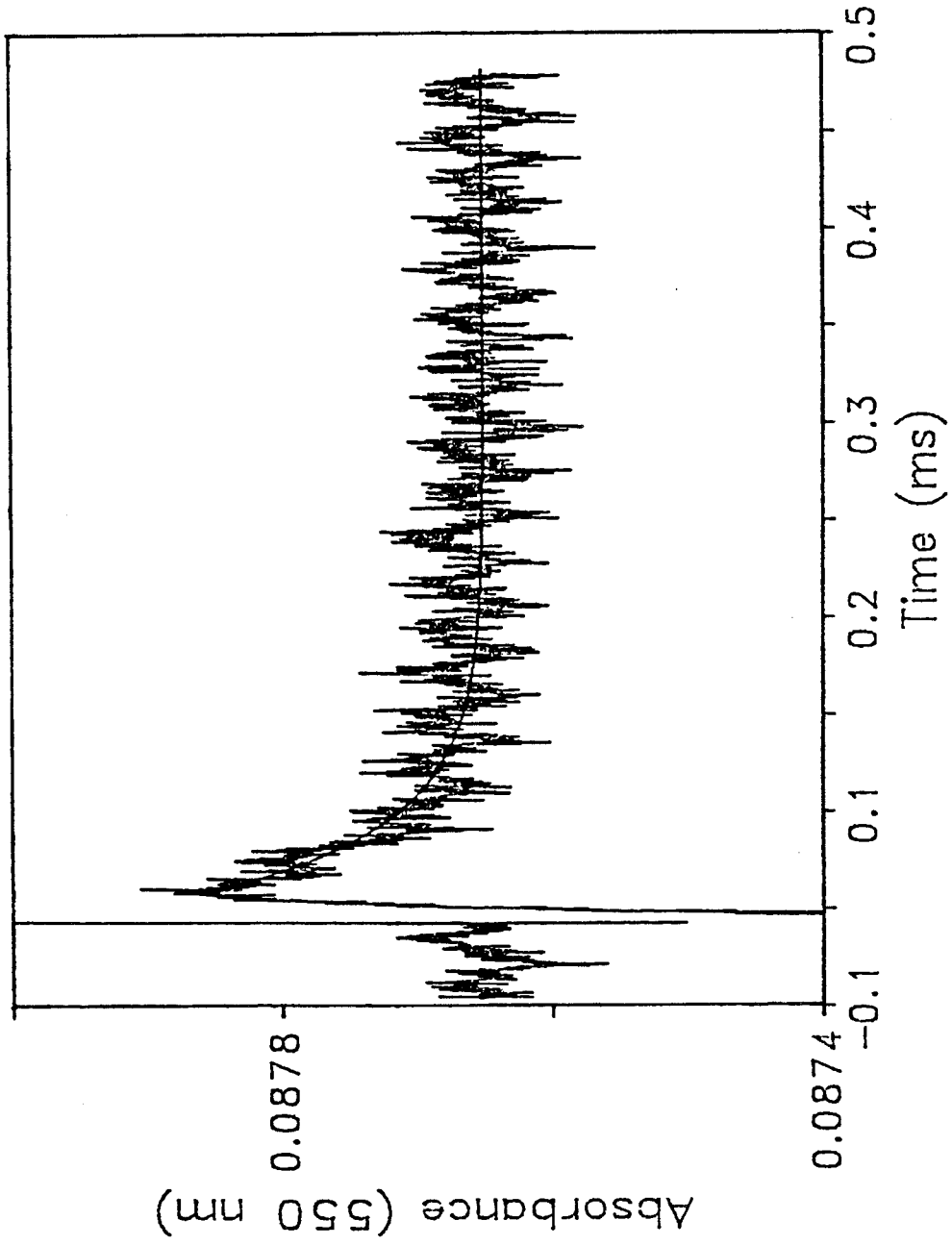

451 nm
Heme
Lys86
Heme
450 nm
0
20
40
60
80
100
120
Time (min)

Figure III-6: Intramolecular electron transfer of $Ru(bpy)_2(dcbpy)$-cytochrome c derivative modified at lysine 86 (20 μM in 100 mM phosphate, pH 7.0). The absorption transient was followed at 550 nm following a laser pulse.


Absorbance (550 nm)
0.0878
0.0874
−0.1
0.1
0.2
0.3
0.4
0.5
Time (ms)

cytochrome c heme in the complex. In these experiments aniline and 3CP were used as sacrificial redox agent to reduce Ru^{III} and prevent the back reaction k_2 (Scheme I). When the cytochrome c:CcO complex was preformed at low ionic strengths, a biphasic oxidation of cytochrome c was observed (Figure III-7a). The absorbance transients were fitted to the sum of two exponentials: $A=A_0\{f\exp(-k_f t) + (1-f)\exp(-k_s t)\}$ with pseudo-first-order rate constant of 560 s^{-1} for the fast phase and 114 s^{-1} for the slow phase. The fast phase has an amplitude corresponding to 45% of the total signal change (Δ[cyt c^{2+}] = 0.048μM). The corresponding transient reduction of CcO is shown in Figure III-7b. The reduction of the low potential cytochrome a, observed at wavelength of 604 nm, occurs at an observed pseudo-first-order rate constant of 2.3 x 10^4 s^{-1}, corresponding to Δ[cyt a^{2+}] = 0.032μM. The transient absorbance of Cu_A was monitored at 830 nm. We observed that the transient signal at 830 nm was very weak (data not shown). So the transient data at 830 nm are not used to obtain information from.

Ionic Strength Effects on the Kinetics. The effect of ionic strength on the kinetics of electron transfer between 10 μM Ru-86-cytochrome c and 10 μM cytochrome c oxidase are summarized in Figure III-8. The ionic strength of the solution was adjusted by sodium chloride. The observed first-order rate constant of cytochrome a reduction remained unchanged as the ionic strength was increased from 10 mM to 30 mM. However, further increasing of the ionic strength resulted in a decrease in the rate constant.

Discussion.

Figure III-7a: Intracomplex electron transfer from Ru-86-cytochrome c to cytochrome c oxidase. The solution contained 10 μM Ru-86-cytochrome c and 15 μM cytochrome c oxidase in 5 mM sodium phosphate, pH 7.0, 0.1% dodecyl maltoside, 10 mM aniline, and 1 mM 3CP. The oxidation of cytochrome c was followed at 550 nm. The smooth lines give the optimized fits to the experimental transients.

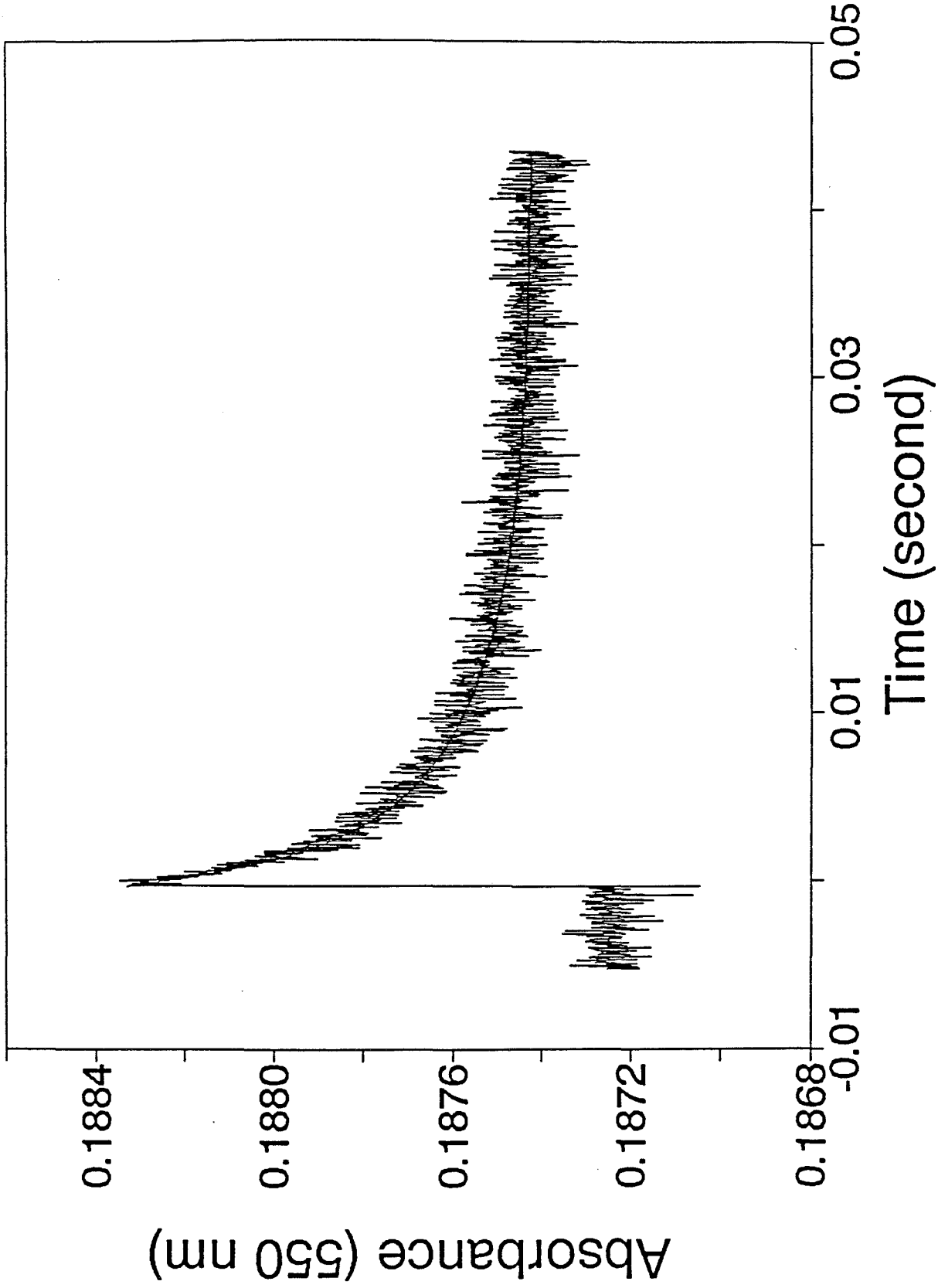

Absorbance (550 nm)
0.1884
0.1880
0.1876
0.1872
0.1868
-0.01
0.01
0.03
0.05
Time (second)

Figure III-7b: Intracomplex electron transfer from Ru-86-cytochrome c to cytochrome c oxidase. The solution contained 10 μM Ru-86-cytochrome c and 15 μM cytochrome c oxidase in 5 mM sodium phosphate, pH 7.0, 0.1% dodecyl maltoside, 10 mM aniline, and 1 mM 3CP. The reduction of cytochrome a in cytochrome c oxidase was detected at 604 nm. The smooth lines give the optimized fits to the experimental transients.

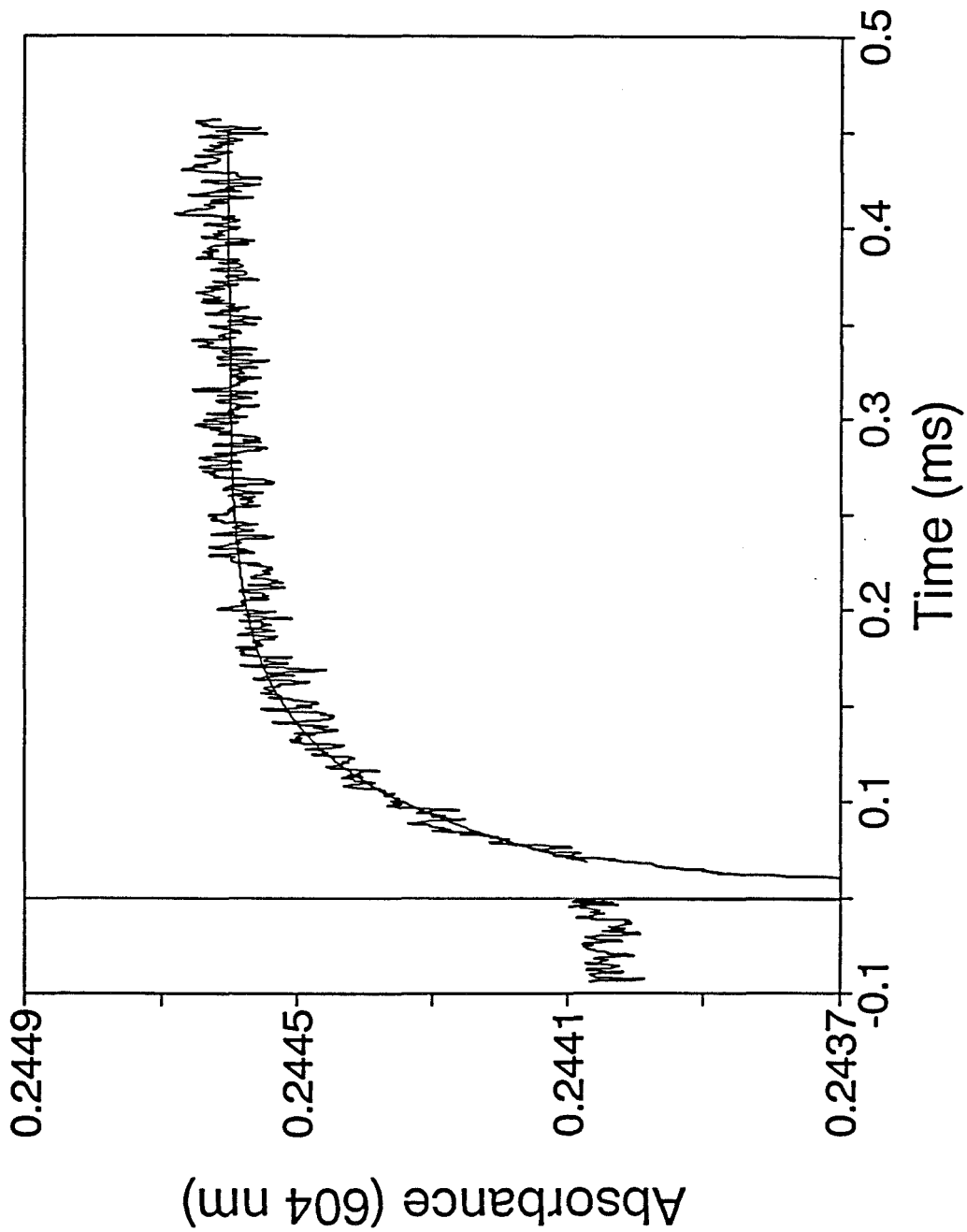

0.2449
0.2445
0.2441
0.2437
Absorbance (604 nm)
-0.1
0.1
0.2
0.3
0.4
0.5
Time (ms)

Figure III-8: Ionic strength effects on the rate constant k_{obs} for reduction of cytochrome a in cytochrome c oxidase by Ru-86-cytochrome c.

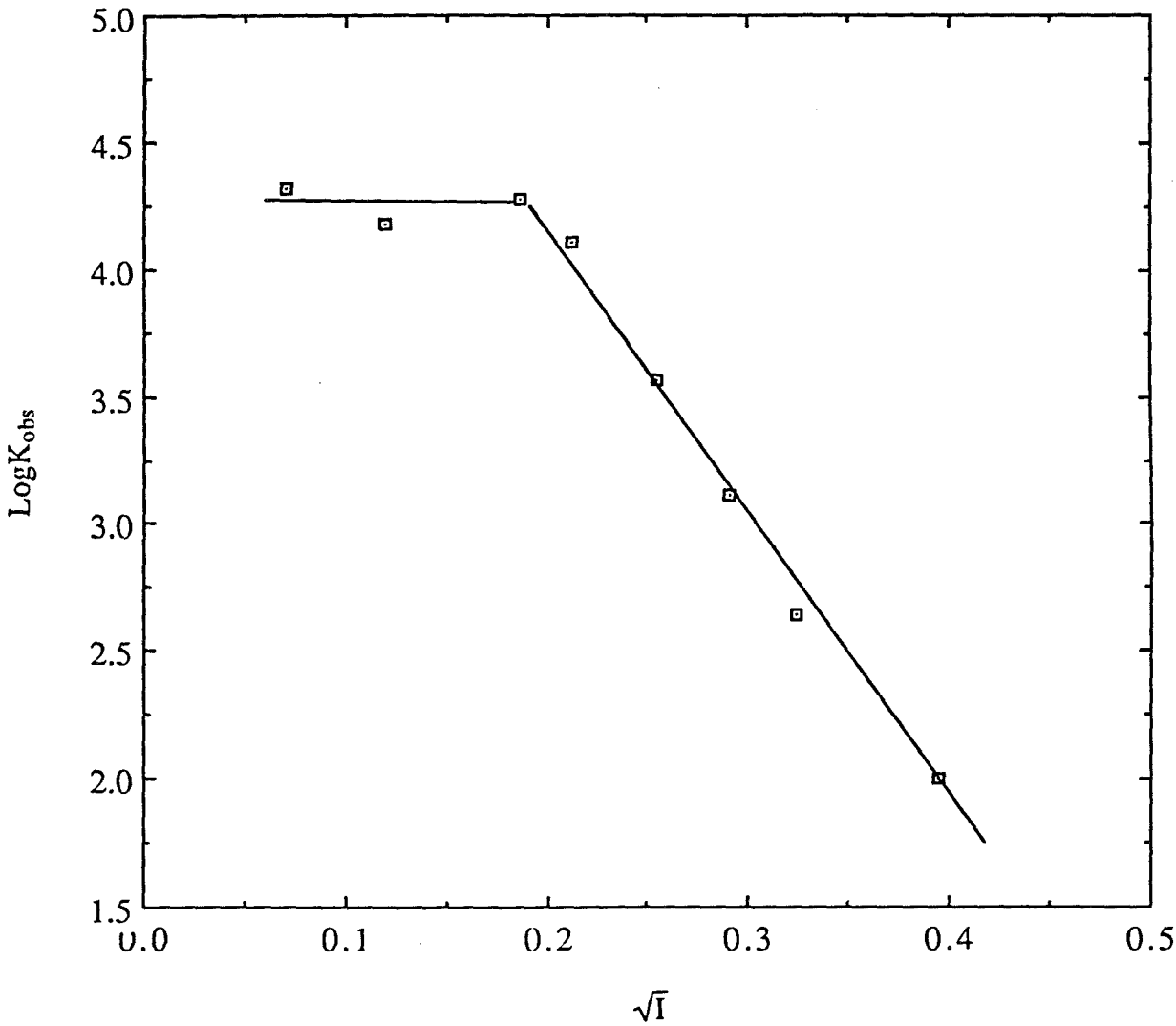

5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
0.0
0.1
0.2
0.3
0.4
0.5
LogK$_{obs}$
$\sqrt{I}$

Photo-Induced Electron Transfer of Ruthenium-86-Cytochrome c.

The convolution function at 550 nm is $i(Fe^{II}, 550nm) = A[e^{-(k1+kd)t} - e^{k2t}]$. From the transition at 556.5 nm, we obtained that the item of $e^{-(k1+kd)t}$ deacys extremely fast with a rate constant of about 10^6 s^{-1}. Therefore, at our slow time scale measurement, the process represented by the first item in the above equation has been over and does not exit in the transient we observed. Accordingly, the fitting of the 550 nm transient gives a general information of k_2, which is 2.8×10^4 s^{-1}.

Intracomplex Electron Transfer between Cytochrome c and CcO.

The ruthenium modified cytochrome c retains the overall net charge as native cytochrome c, consequently, it forms 1:1 complexes with CcO at low ionic strength on the basis of electrostatic interaction as in the case of native cytochrome c and CcO. According to the more recent studies by Pan et al. (1993) on derivatives modified at lysine residues remote from the binding domain of cytochrome c, the rate constant for electron transfer from the cytochrome c heme to Cu_A is 10^4 s^{-1} to 10^5 s^{-1} at low ionic strengths, from which electron is then transferred to cytochrome a with a rate constant of about 10^4 s^{-1}. But for derivatives modified at lysines 13 and 27, the intracomplex electron transfer rate constants range from 1000 to 6000 s^{-1} and no apparent reduction of Cu_A was observed. They concluded that the sequence of intracomplex electron transfer is from the photoreduced heme of ruthenated cytochrome c to Cu_A in CcO; Cu_A then transfers electron to cytochrome a. The decreased rate constants obtained from lysine 13 and 27 derivatives in electron transfer experiments are attributed to the alteration in the binding orientation caused by the ruthenium complexes.

In the present study, photoexcitation of the complex between $Ru(bpy)_2(dcbpy)$-Lys 86 cytochrome c and CcO results in rapid reduction of the heme group of cytochrome c, followed by transfer of the electron to CcO. Since lysine 86 is located in the interaction domain, the large ruthenium moiety is expected to alter the binding orientation of cytochrome c to CcO. However, the rate of intracomplex electron transfer is not significantly slower than those in the other cytochrome c derivatives. It is surprising that this derivative is active as it is, considering the large size of the ruthenium complex. Obviously, the big moiety of the ruthenium complex in the binding domain does not hinder the electron transfer. We think that there are actually three phases of Ru-86-cytochrome c oxidation, which should be observable at 550 nm. The fastest phase involves electron transfer from reduced cytochrome c to CcO with a rate constant greater than 10^5 s^{-1}. This rapid rate is too fast to be resolved by our instrument. We believe that the rapid phase exists since we observe the rate of electron transfer to cytochrome a is 2.3×10^4 s^{-1}. The other two phases are slow and are being monitored. The three different kinetic phases of Ru-86-cytochrome c oxidation suggests three different binding orientations, one orientation allowing rapid electron transfer and the other two corresponding to much slower electron transfer.

Studies of intracomplex electron transfer have shown that lysines 13 and 27 derivatives transfer electron to CcO at a much slower rate than other derivatives. It is believed that the slow rates stem from lysines 13 and 27 involved in the binding domain between cytochrome c and CcO. Previous chemical modification studies demonstrate that lysines 13, 86 and 87 are crucial in the binding of cytochrome c with CcO (Pettigrew and Moore, 1987). Compared with other derivatives, the transient absorbance of Ru-86-

cytochrome c at 830 nm is pretty weak and limited by light scattering artifacts. So we surmise that Cu_A is not the primary electron acceptor with the Ru-86-cytochrome c derivative. It is possible that the ruthenium complexes are in the binding domain and alter the binding orientation in cytochrome c : CcO complexes. This alteration may allow direct electron transfer from Ru-86-cytochrome c to cytochrome a in CcO at low ionic strength. As a result, the Cu_A is no longer the primary electron input port; the cytochrome a now functions as the electron acceptor. Cytochrome a in CcO can therefore be employed as a back-up for Cu_A to accept electron from ferrocytochrome c. The transient at 550 nm we observed is very slow. The slow phase can be fitted by biphasic kinetics. We suggest that these two slow kinetics correspond to two different binding orientations. These two orientations are not aligned properly, leading to slow electron transfer from Ru-86-cytochrome c to CcO.

Ru-86-cytochrome c derivative is actually expected to have a slow intracomplex electron transfer rate. The bulky ruthenium complex at the binding domain should alter the binding orientation. This change should modify the electron transfer rate if only Cu_A can function as the primary electron acceptor. But the rate of electron transfer from Ru-86-cytochrome c to cytochrome a in CcO, we observed, is 2.3×10^4 s^{-1} similar to those derivatives in which the covalently labeled ruthenium complexes do not affect the binding domain. It is clear that once Ru-86-cytochrome c forms complexes with CcO, the ruthenium complex alters the binding orientation. The new orientation favors transferring electron to cytochrome a instead of Cu_A. The electron transfer pathway to cytochrome a corresponding to the new orientation appear to be very efficient. Thus it seems that cytochrome a can function well as Cu_A as the initial electron acceptor.

We observe that the rate constant for reduction of cytochrome a remains constant over the range of ionic strength between 10 to 30 mM (Figure III-8). Above 30 mM in ionic strength, the rate constant decreases. Under these conditions, we expect dissociation of the complex formed between the redox partners and the kinetics become bimolecular. The plot gives the minimum ionic strength needed to dissociate the complex.

In conclusion, the kinetics of electron transfer from cytochrome c to CcO can be extremely complex. By varying the site of the modification, different docking complexes can be formed at low ionic strengths, corresponding to different orientations of cytochrome c vis a vis CcO in the docking pocket. It is apparent that the ferrocytochrome c can transfer its electron directly into either Cu_A or cytochrome a, with different rates, depending upon the orientation of ferrocytochrome vis a vis the oxidase in the docking pocket. If multiple docking complexes are formed for a given cytochrome c derivative, and the electron transfer kinetics to CcO within each of the docking complexes is rapid relative to the time scale of interchanges among the various docking complexes, multiphasic electron input into the oxidase is expected for each electron acceptor, as observed here. However, under high ionic strengths, the docking complexes cannot be so tight, and we must have:

$$k_{c\rightarrow a}^{\text{direct et}} = \int P(r)\, k_{c\rightarrow a}(r)\, dr$$

$$k_{c\rightarrow A}^{\text{direct et}} = \int P(r)\, k_{c\rightarrow A}(r)\, dr$$

where P(r) is the distribution of approach of the cytochrome c vis a vis the oxidase in the docking pocket, and $k_{c\text{-}a}(r)$, $k_{c\text{-}A}(r)$ refer to the intrinsic electron transfer rate constants for the two electron input ports for a given

orientation. Clearly, P (r) is a function of the details of the docking within the docking pocket.

In a previous study, Pan et al. (1991) examined electron input into a derivative of CcO in which the Cu_A center had been depleted. Their studies demonstrated that the rate of cytochrome a reduction was significantly reduced in the Cu_A-depleted form of the enzyme. However, with the Cu_A-depleted enzyme, cytochrome a can also accept an electron directly from cytochrome c, albeit at a slower rate. The results of the present study clearly demonstrate that there may exist two distinct electron-input ports for electron transfer from cytochrome c to CcO, with the more facile one proceeding via Cu_A.

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IV Intramolecular Electron Transfer in CO-Bound Mixed-Valence Cytochrome c Oxidase Following CO Photolysis

Abstract.

Internal electron transfer in bovine cytochrome c oxidase was initiated by CO photolysis of the CO-bound mixed-valence form of the enzyme. Transient absorption spectroscopy was used to monitor changes in the redox states of the metal centers in the enzyme brought about by electron re-equilibration. Upon CO photodissociation, reduced high spin cytochrome a_3 was generated in less than 0.1 μsec, and a portion of the reduced cytochrome a_3 was reoxidized with biphasic rate constants of $k_1 = 1.0 \times 10^6\ s^{-1}$ and $k_2 = 7.8 \times 10^4\ s^{-1}$. Concomitant reduction of cytochrome a was also observed with biphasic rate constants of $k_1 = 1.6 \times 10^6\ s^{-1}$ and $k_2 = 9 \times 10^4\ s^{-1}$. The stoichiometry of cytochrome a_3 oxidized to cytochrome a reduced was found to be close to 1:1. Contrary to similar studies in the literature, no reduction of Cu_A was observed. As a control, no transient absorption changes corresponding to electron transfer was observed in the CO-inhibited fully reduced form of the enzyme. These results indicate that there is significant electron reequilibration only between cytochrome a_3 and cytochrome a upon photolysis of CO-bound mixed-valence enzyme.

Introduction.

Cytochrome c oxidase, the terminal enzyme in the mitochondrial respiratory chain, catalyzes the transfer of electrons from ferrocytochrome c to molecular oxygen and reduces the dioxygen to water (Wikström et al., 1981; Chan & Li, 1990). Concomitant with this electron transfer, cytochrome c oxidase also pumps protons from the matrix side to the cytosol side of the membrane. In this way, an electrochemical gradient is generated for synthesis of ATP (Krab & Wikström, 1987).

The enzyme contains four redox active metal centers; two heme A prosthetic groups (cytochrome a and cytochrome a_3) and two copper ions (Cu_A and Cu_B) (Blair, et al., 1983). It is generally agreed that cytochrome a and/or Cu_A serve as the primary electron input sites to accept the electrons from ferrocytochrome c (Pan et al., 1991). Cytochrome a_3 and Cu_B form the binuclear center where dioxygen is activated and reduced to water (Wikström & Casey, 1985; Blair et al., 1986).

Toward understanding the molecular mechanism of redox-linked proton pumping in cytochrome c oxidase, several groups of investigators have begun to examine the details of the internal electron transfers in the enzyme (Boelens et al., 1982; Brzezinski & Malmström, 1987; Morgan et al., 1989; Olivberg & Malmström, 1991). Since four electrons are required to reduce one molecule of oxygen to water by the enzyme, the flow of the electrons among the four redox metal centers is very complex and still not well understood. For example, it is still not clear whether only Cu_A accepts electrons directly from ferrocytochrome c and whether only cytochrome a

transfers the electrons to the dioxygen binding site. Determination of the electron transfer routes in cytochrome c oxidase molecule under varying stages of turnover of the enzyme as well as various states of energetization of the mitochondrion are of specific interest because the information may lead to the possible resolution of the redox-linked proton pumping mechanism in cytochrome c oxidase.

CO-bound cytochrome c oxidase provides an unique system for the mapping of some of these internal electron transfer routes under a subset of conditions. It is well known that various forms of the enzyme in which the four redox centers are in different redox states can be generated after CO-photodissociation from the enzyme. Depending on the number of reducing equivalent within the protein, photolysis of CO can cause electron redistribution among all the redox active metal centers in cytochrome c oxidase. Several groups have shown, for example, that CO-photodissociation from the CO-inhibited mixed-valence oxidase results in partial reduction of cytochrome a and/or Cu_A. Observation of this electron "backflow" is one of the few direct protocols by which internal electron transfer in cytochrome c oxidase can be studied.

However, these experiments have not always led to consistent findings. Boelens et al. (1982) reported electron transfer reequilibration (k_{app}=7,000 s^{-1}) between the binuclear center and Cu_A after photodissociation of CO from mixed-valence enzyme. Later Brzezinski and Malmström (1987) observed the same kinetic equilibrium, but reported a faster rate constant (k_{app}=14,000). In 1991, Oliveberg and Malmström repeated the same experiment for both the two- and three- electron-reduced cytochrome c

oxidase. Based on these updated results, they modified and extended their earlier conclusions to include a second equilibration between cytochrome a and Cu_A following the initial reequilibration between cytochrome a_3/Cu_B binuclear center and cytochrome a. While the rapid electron reequilibration between cytochrome a_3 and cytochrome a seems be well established, the involvement of the Cu_A site in the reequilibration remains very much unsettled.

In the present study, we have repeated the same laser transient absorption experiments on CO-bound mixed-valence and CO-bound fully reduced enzymes at the microsecond time scale. Extension of these experiments to shorter times allows us to investigate the internal electron transfers after CO photodissociation more precisely. These new experiments indicate that CO can be photodissociated by laser flash in less than 0.1 μsec in both the CO-bound mixed-valence and CO-bound fully reduced enzymes with a yield in excess of 20%. In the CO-bound mixed-valence enzyme, we find that 30% of reduced cytochrome a_3 generated during the CO photodissociation is reoxidized in a few microseconds. A corresponding reduction of cytochrome a was observed with the same kinetic rate as the reoxidation of cytochrome a_3. The stoichiometry of cytochrome a_3 reoxidized to cytochrome a reduced was found to be 1:1 (molar ratio). No reduction of Cu_A was observed. As a control, no transient absorption signals were detected for the CO-bound fully reduced enzyme upon photolysis.

Experimental Procedures.

Beef heart cytochrome c oxidase was isolated as described by Hartzell & Beinert (1974). Enzyme concentrations were determined spectrophotometrically by using $\Delta\varepsilon$ (reduced minus oxidized = 24 $mM^{-1}cm^{-1}$) at 605 nm. The enzyme preparation was stored at -80 °C in 25mM K-Phos, 0.5% Brij-35, pH 7.8 buffer until used. The enzyme had a turnover number of 500 s^{-1}.

CO-bound mixed-valence cytochrome c oxidase was prepared by incubating resting enzyme with CO at room temperature for overnight (Bickar et al., 1984). The concentration of enzyme was 10 μM in 50 mM NaH_2PO_4, 0.1% laury maltoside, pH 7.4 buffer. The formation of CO-bound mixed-valence cytochrome c oxidase was confirmed by the shift of the Soret absorbance maximum to 430 nm.

CO-bound fully reduced enzyme was prepared as follows: CO was bubbled into a solution of 10 μM cytochrome c oxidase in 50 mM NaH_2PO_4, 0.1% laury maltoside, pH 7.4 buffer through a needle valve for 8 hr, followed by titration with sodium dithionite. Upon addition of the sodium dithionite, a shoulder developed at 444 nm in the absorption spectrum, indicating reduction of cytochrome a. Complete reduction of the enzyme was indicated when the addition of a slight excess sodium dithionite did not cause any further change to the absorption spectra. An HP 8452A UV-visible spectrophotometer was used to monitor the absorbance change.

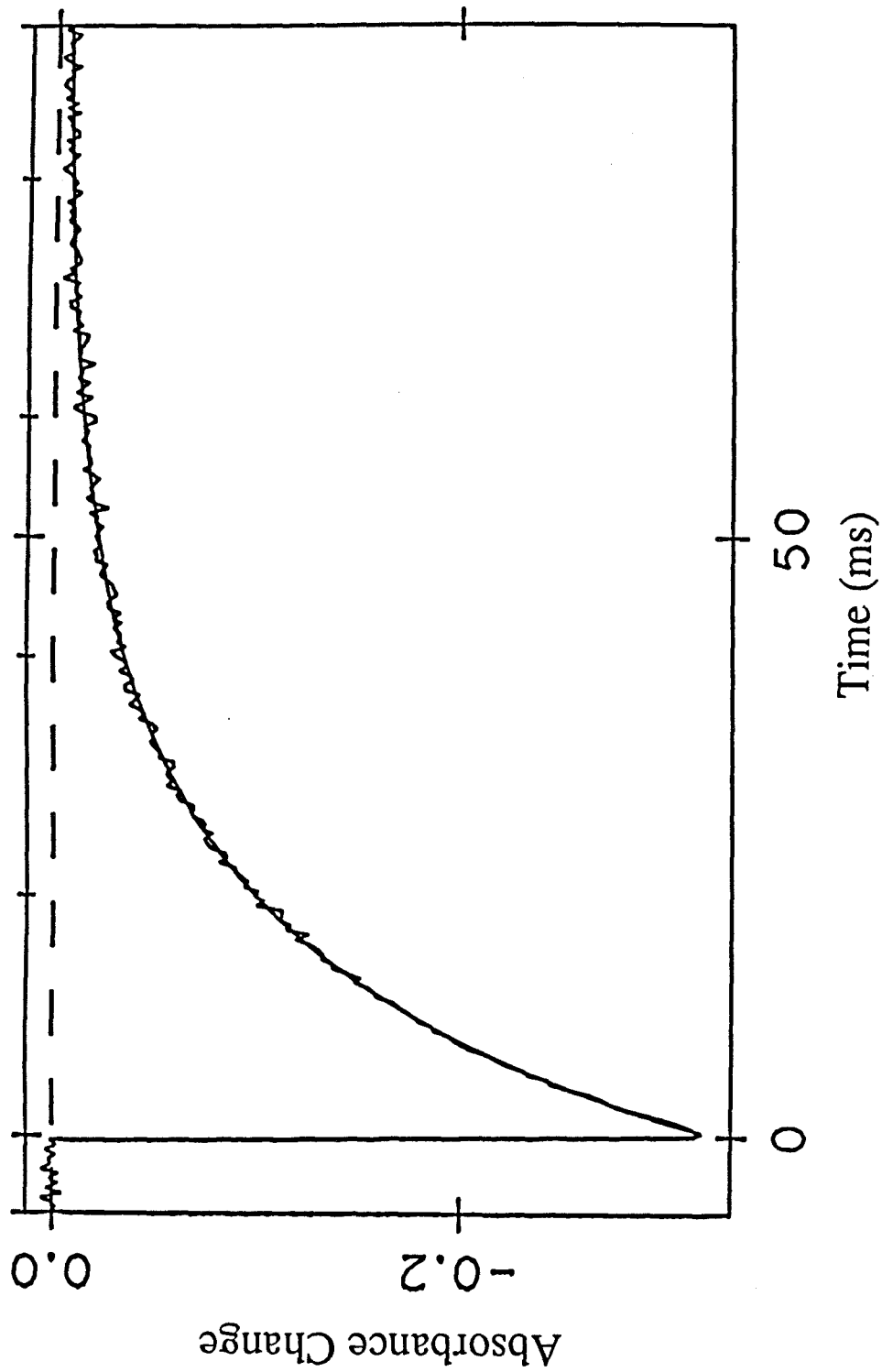
Transient absorption kinetics were measured by flash photolysis of 2 mL solutions contained in a 1-cm glass cuvette. Photolysis was accomplished with a 480-nm pulse from a Lambda Physik FL 3002 dye laser using coumarin

102 dye, which was pumped by a Lambda Physik LPX 210i excimer laser. The laser excitation was run at a repetition rate of 2 Hz and a pulse width of about 25-ns with an energy of about 1.5 mJ. Probe light in the UV-visible region was provided by a 75W xenon arc lamp. The excitation beam was made co-linear with the probe light beam by means of a dichroic mirror and was focused to a spot size of about 1 mm. The transmitted light was passed through an Instruments SA 1680B double monochromator and detected by a photomultiplier tube. The signal was digitized using a Tektronix R710 200 MHz transient digitizer interfaced to a computer. The transient signal was amplified using either a 200 MHz quasi differential amplifier (for kinetic time scale out to 750 μs) or a Lecroy DSP 1402E programmable amplifier (for kinetic time scale out to 10 ms). The redox state of Cu_A was determined by measuring the near IR absorption at 830 nm in Prof. Francis Millett's laboratory (University of Arkansas). The detection system at this wavelength consisted of a tungsten lamp, an 830 nm interference filter with a 10 nm bandpass, and a high sensitivity photodiode detector. Finally, the absorbance transients were analyzed with the KINFIT kinetic program for single- and double-exponential decays supplied by Dr. Jay Winkler of the Beckman Institute at the California Institute of Technology.

Results.

Both the CO-bound mixed-valence and CO-inhibited fully reduced enzymes display very fast CO dissociation upon laser photolysis. When the CO-dissociation kinetics of CO-bound mixed-valence enzyme is observed at 430 nm, the characteristic wavelength of Fe_{a3}^{2+}-CO (Figure IV-1), we find that the CO-photodissociation is completed in less than 0.1 μs, which is too fast to

Figure IV-1: Photoinduced CO-dissociation and the subsequent CO-recombination of the CO-bound mixed-valence cytochrome c oxidase monitored at 430 nm. The solution contains 10 μM CO-bound mixed-valence enzyme in 50 mM NaH_2PO_4, 0.1% lauryl maltoside, pH 7.4 buffer. The fit shown as the solid line is a double exponential equation $A = A_0\{f\exp(-k_1t) + (1-f)\exp(-k_2t)\}$ with $k_1 = 123\ s^{-1}$ and $k_2 = 57\ s^{-1}$. The slow phase has an amplitude corresponding to 90% of the total signal change.

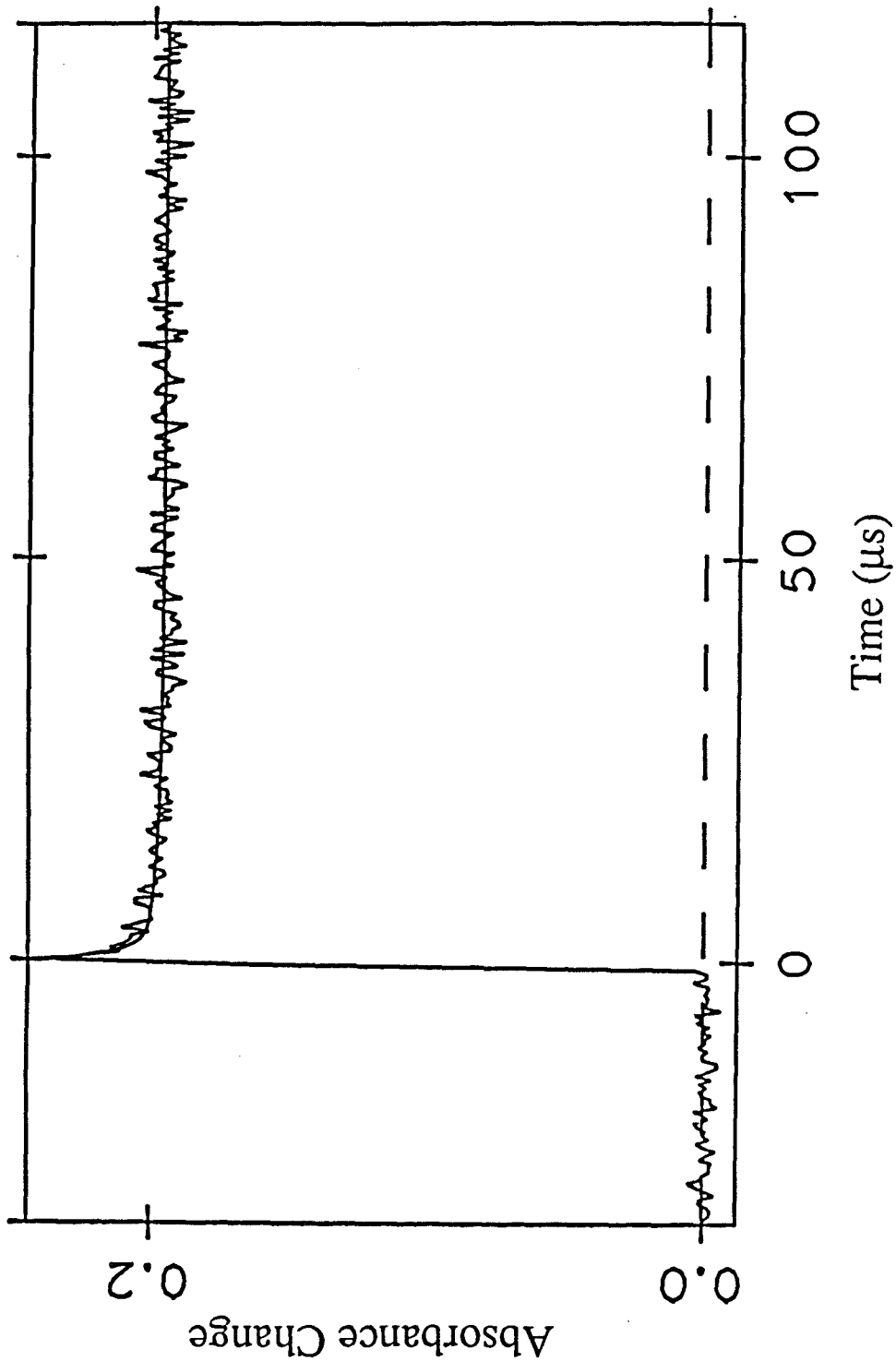

Absorbance Change
0.0
-0.2
0
50
Time (ms)

be resolved by our instrumentation. Following CO-photodissociation, CO recombination occurs, as evidenced by the recovering of the transient absorbance back to the original level. The kinetics of CO recombination is biphasic with k_1 = 123 s^{-1} and k_2 = 57 s^{-1}. A similar kinetic behavior is observed for the CO-inhibited fully-reduced enzyme. Thus the redox states of Cu_A and Fe_a do not affect the photodissociation of CO from cytochrome a_3 as well as the subsequent CO recombination. These results are in agreement with the previous reports of Boelens et al. (1982) and Morgan et al. (1989).

The absorption spectra of cytochrome c oxidase is attributable to both cytochrome a and cytochrome a_3. Interpretation of these spectra in terms of cytochrome a and a_3 has been the subject of much controversy and ambiguity over the years (Vanneste, 1966; Lemberg, 1969). However, it is now clearly demonstrated that the reduced – oxidized absorbance of the 604 nm band is mainly due to cytochrome a [$(\varepsilon_a{}^{2+})_{604nm}$ = 40 ± 1 mM^{-1} cm^{-1}; $(\varepsilon_a{}^{3+})_{604nm}$ = 23 ± 1 $mM^{-1}cm^{-1}$; $(\varepsilon_{a3}{}^{2+})_{604nm}$ = 32 ± 1 $mM^{-1}cm^{-1}$; $(\varepsilon_{a3}{}^{3+})_{604nm}$ = 25 ± 1 $mM^{-1}cm^{-1}$] whereas the band at 444 nm contains contributions from both reduced cytochrome a and a_3 in roughly equal proportions [$(\varepsilon_a{}^{2+})_{444\ nm}$ = 56 ± 2 $mM^{-1}cm^{-1}$; $(\varepsilon_{a3}{}^{2+})_{444\ nm}$ = 112 ± 2 $mM^{-1}cm^{-1}$]; Wikström et al., 1981; Blair et al., 1982). Oxidized cytochrome a and a_3 contribute significantly to the 604 nm band, but not at all to the 444 nm absorption (Wikström et al., 1981). These spectroscopic signatures could be exploited to follow the oxidation/reduction of cytochrome a and a_3 in real time.

Figure IV-2 shows the transient absorption observed at 444 nm following CO-photodissociation of the CO-bound mixed-valence oxidase. The rapid transient arises from generation of $Fe_{a3}{}^{2+}$ from $Fe_{a3}{}^{2+}$-CO as a result of

Figure IV-2: Photoinduced transient absorbance changes of CO-bound mixed-valence cytochrome c oxidase observed at 444 nm. The solution is the same as that described in Figure IV-1. The fit shown is a double exponential equation $A = A_0\{f\exp(-k_1t) + (1-f)\exp(-k_2t)\}$ with $k_1 = 1.0 \times 10^6\ s^{-1}$ and $k_2 = 7.8 \times 10^4\ s^{-1}$. The fast phase has an amplitude corresponding to 85% of the total signal change.


Absorbance Change
0.0
0.2
0
50
100
Time (μs)

the photodissociation; and the subsequent decay can be attributed to electron reequilibration resulting in reoxidation of Fe_{a3}^{2+} together with concomitant reduction of cytochrome a and/or Cu_A, as previously reported by others (Oliveberg & Malmström, 1991). Using an extinction coefficient of 112 $mM^{-1}cm^{-1}$ for reduced cytochrome a_3 at 444 nm, we calculate that about 2.4 μM Fe_{a3}^{2+} was generated from Fe_{a3}^{2+}-CO. Since the oxidase concentration is about 10 μM, the yield is 24%. A similar generation of Fe_{a3}^{2+} was detected at 444 nm upon CO-photodissociation of the CO-inhibited fully-reduced enzyme. In this case, however, a smaller fraction of the total Fe_{a3}^{2+} was generated by laser flash; more importantly, no further kinetic decay (data not shown) was observed. This confirms that the kinetic decay observed at 444 nm for the CO-bound mixed-valence cytochrome c oxidase represents electron transfer from cytochrome a_3 to either cytochrome a or Cu_A in a subset of the oxidase molecules. If so, the decay signal should be proportional to:

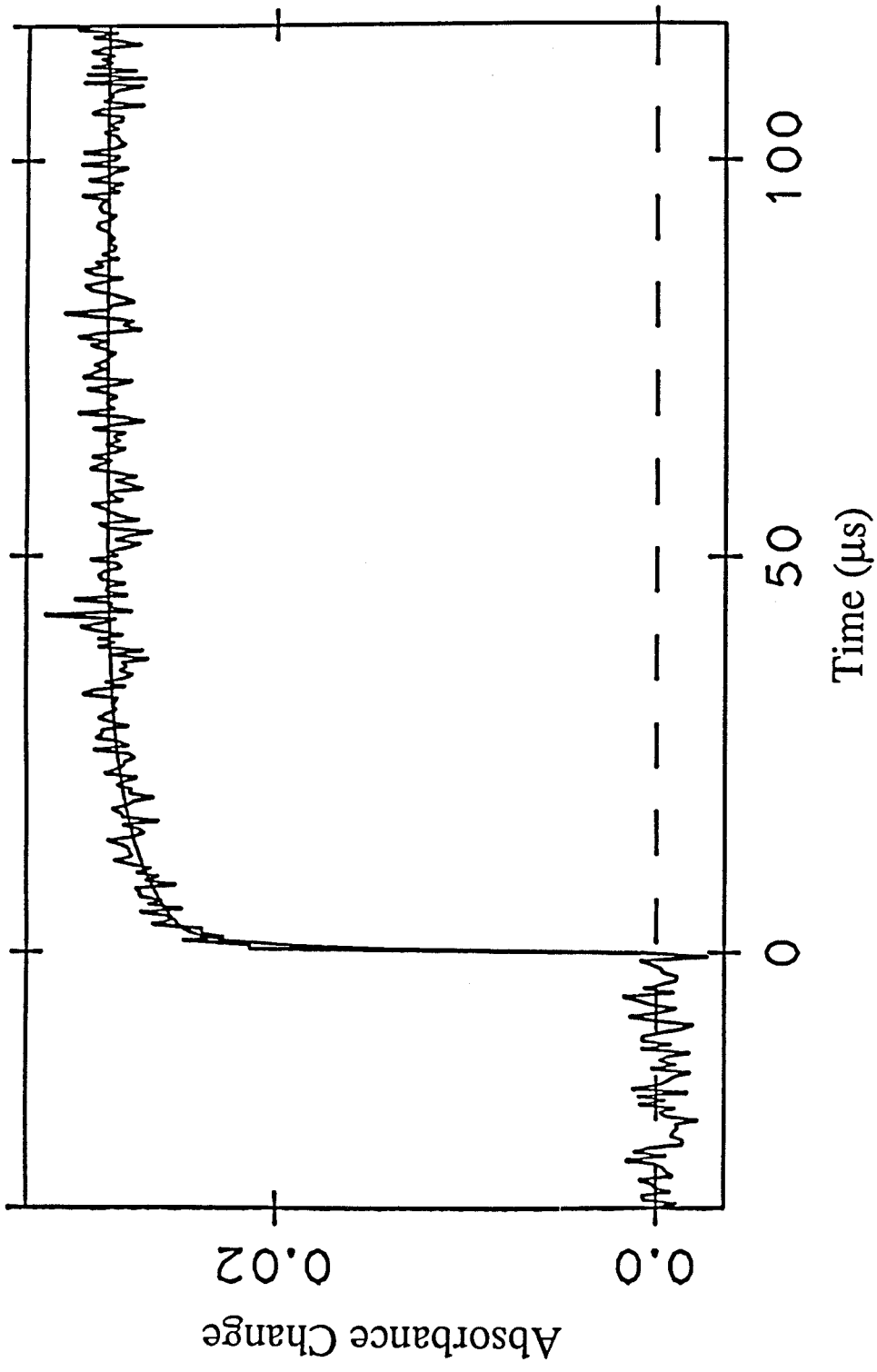
$$(\varepsilon_a{}^{2+})_{444nm}([a^{2+}] - [a^{2+}]_0) + (\varepsilon_{a3}{}^{2+})_{444nm}([a_3{}^{2+}] - [a_3{}^{2+}]_0)$$

$$= (\varepsilon_a{}^{2+})_{444nm}[a^{2+}] - (\varepsilon_{a3}{}^{2+})_{444nm}([a_3{}^{2+}]_0 - [a_3{}^{2+}]).$$

The observed decay kinetics at 444 nm is biphasic and can be fitted well by two exponentials. A rate constant of 1.0×10^6 s^{-1} was obtained for the fast phase with an amplitude corresponding to 85% of the total signal change. The slow phase was fitted to a rate constant of 7.8×10^4 s^{-1}.

To determine whether only one or both of the low potential centers (Cu_A and cytochrome a) is involved in the electron reequilibration with Fe_{a3}^{2+}, we monitored the transient absorption at 604 nm. The extinction coefficient for reduced – oxidized cytochrome a at 604 nm is 17 $mM^{-1}cm^{-1}$ and the corresponding extinction coefficient for cytochrome a_3 is 7 $mM^{-1}cm^{-1}$ (Blair et al., 1982). Figure IV-3 shows the reduction of cytochrome a together

Figure IV-3: Absorbance changes observed at 604 nm following CO photolysis of CO-bound mixed-valence cytochrome c oxidase. The solution is the same as described in Figure IV-1. The fit shown is a double exponential equation $A = A_0\{f\exp(-k_1t) + (1-f)\exp(-k_2t)\}$ with $k_1 = 1.6 \times 10^6\ s^{-1}$, $k_2 = 9.0 \times 10^4\ s^{-1}$. The fast phase has an amplitude corresponding to 82% of the total signal change.


Absorbance Change
0.0
0.02
0
50
100
Time (μs)

with partial reoxidation of cytochrome $\underline{a}_3$ in the CO-bound mixed-valence enzyme as detected at 604 nm. The kinetic trace is also biphasic; in addition, the time scale of the reduction of cytochrome $\underline{a}$ is comparable to that for the reoxidation of Fe_{a3}^{2+}. A rate constant of 1.6×10^6 s^{-1} was obtained for the fast phase with an amplitude accounting for 82% of total signal change. The rate constant of the slow phase is 9×10^4 s^{-1}. Again, no transient absorbance change was detected for the CO-inhibited fully reduced enzyme at this wavelength. These results confirm that there is an electron reequilibration between cytochrome $\underline{a}_3$ and cytochrome $\underline{a}$ upon photolysis of CO-bound mixed-valence enzyme. If so, the signal should be proportional to:

$$(\varepsilon_{a3}^{2+} - \varepsilon_{a3}^{2+}{}_{-CO})_{604nm}[a_3^{2+}]_0 + (\varepsilon_a^{2+} - \varepsilon_a^{3+})_{604nm}([a^{2+}] - [a^{2+}]_0) + (\varepsilon_{a3}^{3+} - \varepsilon_{a3}^{2+})_{604nm}([a_3^{3+}] - [a_3^{3+}]_0).$$

The electron transfer rates determined at the two observation wavelengths show excellent correspondence between the reoxidation of ferrocytochrome $\underline{a}_3$ and the reduction of cytochrome $\underline{a}$.

The stoichiometry of the electron transfer from cytochrome $\underline{a}_3$ to cytochrome $\underline{a}$ and Cu_A as well as the extent of the electron reequilibration could be deduced from the amplitudes of the transient absorptions observed at 444 and 604 nm. At 444 nm, both cytochromes absorb at this wavelength in their reduced forms only. If Cu_A does not participate in the electron reequilibration, the amplitude of the transient signal at this wavelength should be proportional to:

$$[(\varepsilon_a^{2+} - \varepsilon_{a3}^{2+})_{444nm}]([a_3^{2+}]_0 - [a_3^{2+}]_\infty) = (\varepsilon_a^{2+} - \varepsilon_{a3}^{2+})_{444nm}][a^{2+}]_\infty.$$

Since the extinction coefficients for the two cytochromes are: 112 ± 2 $mM^{-1}cm^{-1}$ for cytochrome $\underline{a}_3$ and 56 ± 2 $mM^{-1}cm^{-1}$ for cytochrome $\underline{a}$ (Vanneste, 1966; Blair et al., 1982), $[\varepsilon_a^{2+} - \varepsilon_{a3}^{2+}]_{444nm} = -56$ $mM^{-1}cm^{-1}$; and we deduce from the

amplitude of the decay in Figure IV-2 that the amount of Fe_{a3}^{2+} reoxidation, i.e., $([a_3^{2+}]_0 - [a_3^{2+}]_\infty)$, or Fe_a^{3+} reduction, i.e., $[a^{2+}]_\infty$, is 0.7 μM.

The transient absorbance at 604 nm, also includes contributions from both cytochromes. Again, if Cu_A is not involved in the electron reequilibration, the amplitude of the transient signal is proportional to:

$$(\varepsilon_a^{2+} - \varepsilon_a^{3+})_{604nm}([a^{2+}]_\infty) + (\varepsilon_{a3}^{3+} - \varepsilon_{a3}^{2+})_{604nm}([a_3^{2+}]_0 - [a_3^{2+}]_\infty) + [(\varepsilon_{a3}^{2+}) - \varepsilon_{a3^{2+}\text{-CO}}]_{604nm}[a_3^{2+}]_0$$

$$= [(\varepsilon_a^{2+} - \varepsilon_a^{3+}) + (\varepsilon_{a3}^{3+} - \varepsilon_{a3}^{2+})]_{604nm}([a_3^{2+}]_0 - [a_3^{2+}]_\infty) + [(\varepsilon_{a3}^{2+}) - \varepsilon_{a3^{2+}\text{-CO}}]_{604nm}[a_3^{2+}]_0.$$

The first contribution arises from the reduction of cytochrome a, the second contribution arises from the reoxidation of cytochrome a_3. The extinction coefficient for reduced – oxidized cytochrome a at 604 nm is 17 ± 1 $mM^{-1}cm^{-1}$ and the corresponding extinction coefficient for reduced – oxidized cytochrome a_3 at 604 nm is 7 ± 1 $mM^{-1}cm^{-1}$ (Blair et al., 1982). Accordingly, we predict that a total absorbance change of 0.007 would be generated due to back electron transfer from cytochrome a_3 to cytochrome a. This is a lower limit due to the difficulty in deconvoluting the 444 nm transient to locate the absorbance immediately following the CO-photodissociation. In any case, this is very close to the absorbance change of 0.010 ± 0.002 (upper limit) inferred from the transient at 604 nm after correcting for the absorbance from Fe_{a3}^{2+} following the CO-photodissociation from cytochrome a_3 [$(\varepsilon_{a3}^{2+})_{604nm} = 32 \pm 1$ $mM^{-1}cm^{-1}$]. The total observed absorbance change is 0.028, of which the absorbance from Fe_{a3}^{2+} accounts for $32mM^{-1}$ x (0.23 ± 0.02) x 2.4 x 10^{-3}mM = 0.018 ± 0.002 (lower limit). Here we have assumed as others that CO-photodissociation causes a 23 ± 2% increase in the enzyme's absorbance at 604 nm (Wikström et al., 1981). The outcome of this analysis is that the

stoichiometry of Fe_{a3}^{2+} reoxidized to Fe_a^{3+} reduced upon photolysis of CO-bound mixed-valence cytochrome c oxidase is close to 1:1 (molar ratio), indicating that within the error limits of the experiments, the electrons are transferred only to cytochrome a from Fe_{a3}^{2+} during this internal electron transfer process. The details of the above analysis are summarized in Table I.

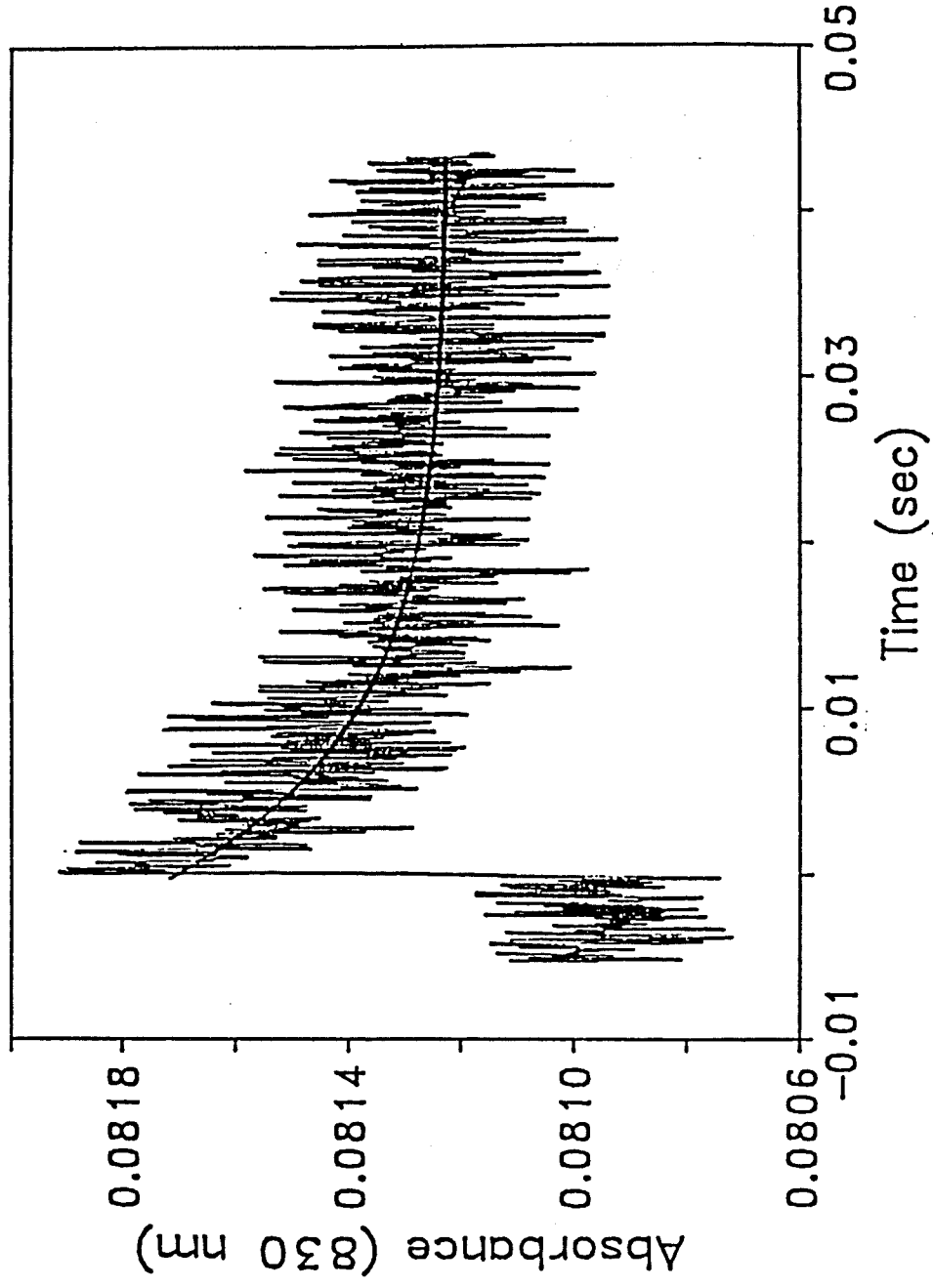
To verify whether the electrons are transferred beyond cytochrome a to Cu_A as suggested by earlier studies (Oliveberg & Malmström, 1991), the reduction of Cu_A was also monitored at 830 nm following photodissociation of CO-bound mixed-valence cytochrome c oxidase. The observed 830 nm transient is depicted in Figure IV-4. We observe an initial transient increase in absorbance followed by a return to its original level with a rate constant of about 110 s^{-1}. However, the pattern of the transient absorption does not correspond to a change in the redox state of Cu_A. Since Cu_A in the CO-bound mixed-valence enzyme is already in the oxidized state, only a decrease in the 830 nm absorption is possible with electron input into this site. Instead, an increased absorption is observed upon CO-photodissociation. Moreover, the rate of the subsequent decay at 830 nm is extremely slow compared with the electron reequilibration rates monitored at 444 and 604 nm. The apparent rate of 110 s^{-1} observed here is more akin to the rate of CO-rebinding to cytochrome c oxidase. Morgan et al. (1989) have noted a similar observation in their transient equilibrium studies of the mixed-valence enzyme. They attributed this recovery to a small absorbance contribution at 830 nm (of the order of 0.0004) with dissociation of CO from cytochrome a_3. Following them, we conclude that Cu_A does not participate to any appreciable extent upon the photolysis of CO-bound mixed-valence cytochrome c oxidase.

Table I. Analysis of transient absorption data assuming that Cu_A does not participate in electron reequilibration upon photolysis of CO-bound mixed-valence cytochrome c oxidase.

Wavelength of Transient	444 nm	604 nm
total absorbance change observed for transient	-0.039 ± 0.001	+0.028 ± 0.001
number of micro moles of cytochrome a_3 reoxidized during electron reequilibration	(-0.039/(-56)) x 10^3 = 0.7 μM	
predicted absorbance change at 604 nm due to reoxidation of Fe_{a3}^{2+} and reduction of Fe_a^{3+}		0.7 x 10^{-3} x (17-7) x 1 = 0.007
absorbance change at 604 nm upon photodissociation of CO from Fe_{a3}^{2+} [$\Delta\varepsilon_{a3}^{2+}$ = 32.1 $mM^{-1}cm^{-1}$ x (0.23 ± 0.02)*]		2.4 x 10^{-3} x 32.1 x 0.23 x 1 = 0.018 ± 0.002
total absorbance change at 604 nm predicted		0.018 + 0.007 = 0.025 ± 0.002

* From Wikström et al. (1981).

Figure IV-4: Absorbance changes monitored at 830 nm following CO photodissociation of CO-bound mixed-valence cytochrome c oxidase. The solution is the same as described in Figure IV-1. The fit is a single exponential with an apparent rate constant of $k = 110\ s^{-1}$.


Absorbance (830 nm)
0.0818
0.0814
0.0810
0.0806
−0.01
0.01
0.03
0.05
Time (sec)

Discussion.

The absorbance changes that we have recorded at 444 and 604 nm following photodissociation of the CO-bound mixed-valence cytochrome c oxidase are very fast (the apparent rate constants are of the order of 10^6 s^{-1} for the fast component). Oliveberg and Malmström (1991) reported similar rate constants at these two wavelengths although the fastest rate they obtained was 2 x 10^5 s^{-1}. It has been suggested that photolysis of CO-bound mixed-valence enzyme initiates a back flow of electrons from the cytochrome a_3 and Cu_B binuclear center to cytochrome a and/or Cu_A. This electron reequilibration is caused by changes in the redox potentials of the cytochrome a_3 and Cu_B relative to those of cytochrome a and Cu_A upon CO-photodissociation (Boelens & Wever, 1979; Fiamingo et al., 1982). Since cytochrome a and Cu_A are about 15-20 Å away from the binuclear cluster (Brudvig et al., 1984; Goodman & Leigh, 1987), this back electron transfer is very efficient despite the long distance.

Under our experimental conditions, we find no evidence for back electron transfer from the reduced binuclear center or from cytochrome a to Cu_A following CO-photodissociation of the CO-bound mixed-valence enzyme. We do not observe any transient signal at 830 nm that could be attributed to Cu_A reduction. Our failure to do so cannot be due to the size of the excitation pulse since the flash used was sufficient to flash off 24% of the sample. The excitation pulse is of sufficient intensity and duration to initiate observable (at 830 nm) electron input into Cu_A from ruthenated cytochrome c's (Pan et al., 1993). Moreover, Cu_A reduction should cause a transient decrease of the 830 nm absorbance. However, we observed an initial increase followed by a

recovery back to original level with slow rate of 110 s^{-1}. Accordingly, in this experiment, the observed increase of the absorbance at 830 nm cannot be due to redox state change of Cu_A. Morgan et al. (1989) have previously reported a similar 830 nm absorbance transient in related experiments and have assigned it to a phenomenon associated with CO-photodissociation and recombination. It should be noted that the magnitude of the 830 nm transient would have corresponded to a level of oxidation/reduction of the Cu_A on the order of 0.2 μM ($\Delta\varepsilon$ = 2.00 $mM^{-1}cm^{-1}$; Blair et al., 1983), about 30% of the redox changes observed for cytochrome a_3 and cytochrome a. Thus, further electron reequilibration from cytochrome a to Cu_A of the order of this level should be discernable by the 830 nm absorbance, though the observed transient is of the opposite sign expected. Unfortunately, a further electron reequilibration of this order from cytochrome a to Cu_A would not be readily distinguished at 604 nm, as the expected absorbance change would be within the error of measurement.

Oliveberg and Malmström (1991) have reported the possibility of generating the three-electron-reduced, CO-inhibited sample in their preparation of CO-bound mixed-valence cytochrome c oxidase. According to the studies of Morgan et al. (1989), photo-irradiation of the three-electron-reduced, CO-inhibited form of cytochrome c oxidase initiates a fast electron reequilibration from cytochrome a to Cu_A. The apparent rate of this process is 1.7 x 10^4 s^{-1}. This rate is comparable to the rate of 1.3 x 10^4 s^{-1} reported by Oliveberg and Malmström (1991) and assigned to electron transfer from cytochrome a to Cu_A. Any three-electron-reduced, CO-inhibited cytochrome c oxidase in the CO-bound mixed-valence enzyme will yield an apparent reduction of Cu_A for the latter species.

In the present study, we obtained a stoichiometry of 1:1 molar ratio of cytochrome $\underline{a}_3$ reoxidized to cytochrome $\underline{a}$ reduced following the CO-photodissociation, indicating that only cytochrome $\underline{a}$ and cytochrome $\underline{a}_3$ are involved in the electron reequilibration on the time scale of the experiment. This study provides the first direct experimental evidence in support of this stoichiometry. Other stoichiometries have been reported in previous studies (Oliveberg & Malmström, 1989). The reason for the discrepancy may be the inability to deconvolute the transient signals into their components, due to the poorer time resolution in the earlier experiments.

Based on this work, we conclude that upon photolysis of the CO-bound mixed-valence enzyme, there is significant internal electron back flow from reduced cytochrome $\underline{a}_3$ to oxidized cytochrome $\underline{a}$ only. Of course, we cannot exclude that there is instead electron transfer from Cu_B to cytochrome $\underline{a}$, followed by very rapid re-reduction of Cu_B by cytochrome $\underline{a}_3$. Our results do not allow us to distinguish between cytochrome $\underline{a}_3$ and Cu_B as the physical electron donor to cytochrome $\underline{a}$. If cytochrome $\underline{a}_3$ and Cu_B are in fast redox equilibrium, as it has been proposed, and the redox potential of Cu_B is higher than that of cytochrome $\underline{a}_3$, the electron will appear to come from cytochrome $\underline{a}_3$ even though Cu_B is the actual donor.

Cytochrome $\underline{c}$ oxidase is a redox-linked proton pump. During the course of electron transfer between its redox-active metal centers, cytochrome $\underline{c}$ oxidase undergoes an ordered sequence of redox state changes. The enzyme is also expected to undergo a series of conformational changes during turnover. There have been several models predicting this behavior of

cytochrome c oxidase (Gelles et al., 1986; Wikström et al., 1981). Because of the relaxation of the enzyme conformations, the electron transfer kinetics may be multiphasic. The biphasic kinetics observed may stem from different internal electron transfer rates within two or more subsets of conformations of the enzyme.

Irrespective of the kinetics of electron reequilibration, the extent of the back electron transfer reflects the relative redox potential differences among the redox active metal centers in the protein. The relative proportions of $Cu_B^{1+}a_3^{2+}a^{3+}Cu_A^{2+}$, $Cu_B^{1+}a_3^{3+}a^{2+}Cu_A^{2+}$ and $Cu_B^{1+}a_3^{3+}a^{3+}Cu_A^{1+}$ observed at equilibrium here indicate that the redox potentials of cytochrome a and Cu_A are ~ 60 mV and > 120 mV, respectively, more negative relative to that of cytochrome a_3 in the one-electron reduced form of the enzyme. These values are in reasonable accord with the limits expected for the redox centers at this stage in the turnover of the enzyme.

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V Conformational Changes of Cytochrome c Oxidase: A Fluorescence Study

Abstract.

Conformational changes have been proposed to accompany the redox change and proton translocation cycle of cytochrome c oxidase. In order to identify the conformational changes, beef heart cytochrome c oxidase was singly labeled by N-iodoacetylamidoethyl-1-aminonaphthalene-5-sulfonate (1,5-I-AEDANS) at subunit III. The fluorescence label on cytochrome c oxidase serves as a conformational probe of the enzyme. Spectral changes in the 1,5-I-AEDANS fluorescence spectrum are indicative of conformational changes which signify the alteration of the environment of the cysteine residue on which 1,5-I-AEDANS has been labeled. We have measured the changes in the AEDANS emission intensity when cytochrome c oxidase was at different redox states. The results of our experimental measurements suggest that there is a redox-linked conformational change in cytochrome c oxidase.

Introduction

Cytochrome c oxidase is the terminal enzyme in the mitochondria respiratory chain. In mammals, this enzyme spans the inner mitochondrial membrane and catalyzes the reduction of molecular oxygen to water (Wikström et al., 1981). In this reaction, the electrons are derived from the oxidation of ferrocytochrome c on the cytosol side of the membrane while the protons consumed for the dioxygen reduction reaction are taken up from the mitochondria matrix side. Coupled with this exergonic reaction, cytochrome c oxidase also catalyzes the redox-linked translocation of up to $4H^+$ from the matrix side to the cytosol side of the membrane for each dioxygen molecule reduced (Wikström, 1977; Proteau et al., 1983). The energy derived in this process is used to drive the phosphorylation of ADP to form ATP (Wikström et al., 1981). Each monomer of cytochrome c oxidase is known to contain four redox-active metal centers, two heme A prosthetic groups (cytochrome a and cytochrome a_3) and two copper ions (Cu_A and Cu_B). Cytochrome a_3 and Cu_B together form a binuclear cluster, dioxygen is bound and reduced there. The other two centers, cytochrome a and Cu_A, are electron input ports and mediate the flow of electron from cytochrome c to the dioxygen binding site.

Cytochrome c oxidase belongs to a distinct class of redox-linked proton translocation enzymes. The nature of the linkage between its redox activity and proton pumping is not well understood at the moment. However, it is becoming increasingly clear that there exists some type of communication between the redox active metal centers and the general function of the enzyme. In simple words, this enzyme can be thought of as a molecular

machine which must undergo a series of conformational changes in order to carry out its function as a redox-linked proton pump.

Several types of conformational changes, large or small, have been studied in cytochrome c oxidase. Yamamoto and Okunuki (1970) reported that the reduced enzyme is more susceptible to protease digestion than the oxidized form. A large decrease in the positive ellipticity at 600 nm was observed by circular dichroism spectroscopy when CO bound fully reduced cytochrome c oxidase was converted to the CO bound mixed-valence form (Yong and King, 1970). Cabral and Love (1972) showed by sedimentation studies that reduction of the enzyme resulted in a ~3% increase in the protein volume. They attributed the main volume increase to the reduction of cytochrome a and Cu_A through comparison of the volume changes in the fully reduced and CO bound mixed-valence (only cytochrome a_3 and Cu_B reduced) enzymes. Using zinc cytochrome c fluorescence as a probe for conformational changes in cytochrome c oxidase, a number of investigators have also demonstrated that the reduction of cytochrome a and Cu_A triggers a conformational change in cytochrome c oxidase (Dockter et al., 1978; Vanderkooi et al., 1977; Kornblatt & Luu, 1986; Geren & Millett, 1981; Miki & Takayoshi, 1984; Alleyne & Wilson, 1987). Chan and co-workers (Ellis et al., 1986; Wang et al., 1986) showed that the standard entropies of reduction for both cytochrome a and Cu_A were quite large in the CO bound enzyme ($\Delta S^{0'}_{cyta}$ = -50.8 eu; $\Delta S^{0'}_{CuA}$ = -48.7 eu). This result is consistent with the proposal that reduction of cytochrome a and Cu_A accompanies conformational changes in cytochrome c oxidase.

Using a different approach, i.e. by studying the kinetic behavior of ligand binding to cytochrome c oxidase, several researchers have shown that reduction of cytochrome a and Cu_A leads to a faster ligand binding rate (Van Buuren et al., 1972; Jones et al., 1984; Jensen et al., 1984; Scholes & Malmström, 1986). Based upon their experiments, these authors argued that there may exist two distinct conformations in cytochrome c oxidase: "open" and "closed" states. Reduction of cytochrome a and Cu_A results in "opening" of the dioxygen binding site. The conformational change corresponding to this process has thereafter been termed as "open-closed" transition.

The available experimental evidence do suggest an important role of the conformational changes involved in the catalytic function of cytochrome c oxidase. Some researchers have proposed that the observed conformational changes must be correlated if not directly associated with the proton pumping cycle of cytochrome c oxidase (Jensen et al., 1984). However, there have been few ways to directly observe conformational changes that occur during the enzyme's catalytic cycle. Tryptophan fluorescence would be very useful and possibly important since it allows one to probe the protein conformational changes during the enzyme turnover. The use of tryptophan fluorescence to probe conformational changes in proton pumping system of bacteriorhodopsin has been reported by Jang et al. (1988). The tryptophan fluorescence spectra of cytochrome c oxidase have also been exploited to probe of redox-linked conformational changes (Smith, unpublished results; Li, unpublished data). The only drawback of this approach is that the intensity of the tryptophan fluorescence in cytochrome c oxidase is too weak to allow any clear interpretation of the acquired data. To circumvent this disadvantage, we use cytochrome c oxidase specifically labeled with the fluorescence reagent, N-

iodoacetylamidoethyl-1-aminonaphthalene-5-sulfonate (1,5-I-AEDANS), to observe protein conformational changes that occur during the turnover cycle of cytochrome c oxidase. Evidence obtained so far indicate that the redox state changes of cytochrome c oxidase indeed result in conformational changes which manifest themselves by the changes of the protein fluorescence spectra.

Experimental Procedures

Materials Beef heart cytochrome c oxidase was separated and purified by the method of Hartzell and Beinert (1974). Enzyme concentrations were determined spectrophotometrically by using $\Delta\varepsilon$ (reduced minus oxidized = 24 $mM^{-1}cm^{-1}$) at 605 nm. The enzyme preparation was stored at -80 °C until used. Horse heart cytochrome c (Type VI) and 1,5-I-AEDANS were obtained from Sigma and used without further purification. Lauryl maltoside was purchased from Fluka. 5-Deazariboflavin was a kind gift from Prof. Gordon Tollin (University of Arizona).

CO-bound mixed-valence cytochrome c oxidase was prepared by incubation of the resting enzyme under an atmosphere of CO at room temperature. The formation of CO-bound mixed-valence enzyme is checked by the Soret band absorption maximum at 430 nm, which is the characteristic wavelength of cytochrome a_3-CO. To fully reduce the CO-bound mixed-valence enzyme, microliter aliquots from a stock solution of dioxygen-free sodium dithionite was added. UV-visible spectrum was used to confirm the extent of reduction of the enzyme.

1,5-I-AEDANS labeled cytochrome c oxidase was prepared as described by Pan et al. (1991). A volume of 50 μM native cytochrome c oxidase in 100 mM Tris-Cl, 0.1% lauryl maltoside, pH 8.0 buffer was treated dithiothreitol anaerobically to a final concentration of 4 mM. Then 5 mM 1,5-I-AEDANS was added and the mixture was incubated for 12 h at 4 °C in the dark. Finally, the reaction solution was chromatographed on a 1x20 cm Sephadex G-25 column equilibrated with 50 mM sodium phosphate, 0.1% lauryl maltoside, pH 7.4 to remove excess reagent.

Cytochrome c derivative covalently labeled by ruthenium bis(bipyridine)dicarboxy-bipyridine [$Ru^{II}(bpy)_2(dcbpy)$] at lysine 86 was prepared according to the procedure described by Pan et al. (1988).

Experimental Methods Fluorescence spectra of 1,5-I-AEDANS labeled cytochrome c oxidase 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. The excitation wavelength of 340 nm and emission wavelength of 480 nm were used in experiments. Spectra were obtained at a scan speed of 1 nm/s. Typically 1.5 ml of 1.5-2 μM 1,5-I-AEDANS labeled cytochrome c oxidase were used for fluorescence measurements. 5-Deazariboflavin was prepared in a 100 μM stock solution containing 1 mM EDTA, 110 mM KCl, 5 mM Tris-Cl, 0.1% lauryl maltoside, pH 7.4; and used with solutions containing 4 μM cytochrome c oxidase labeled with 1,5-I-AEDANS and 4 μM cytochrome c.

UV-visible absorption spectra were recorded for each sample before and after fluorescence data acquisition with an HP 8452a diode array spectrophotometer.

Results

Specific Labeling of Cytochrome c Oxidase by 1,5-I-AEDANS Figure V-1 shows the labeling pattern of cytochrome c oxidase by 1,5-I-AEDANS. The fluorescence probe, 1,5-I-AEDANS, labels cytochrome c oxidase exclusively on subunit III. This result is consistent with previous reports (Hall et al., 1988; Pan et al., 1991). It has been presumed that the specific amino acid residue being labeled is Cys-115, one of the exposed cysteines on subunit III (Hall et al., 1988).

Fluorescence Measurements Figure V-2a shows the fluorescence spectrum of 1,5-I-AEDANS labeled cytochrome c oxidase. When the resting form of cytochrome c oxidase is labeled by 1,5-I-AEDANS, the fluorescence spectrum of the labeled protein has an emission maximum at 480 nm and an excitation maximum at 340 nm.

Figure V-2b shows the fluorescence spectrum of CO-bound mixed-valence, 1,5-I-AEDANS labeled cytochrome c oxidase. When the cytochrome c oxidase is put under an atmosphere of CO, cytochrome a_3 and Cu_B binuclear center is bound with CO and becomes reduced upon the subsequent reaction with CO. At the mean time, the other two centers, cytochrome a and Cu_A, remain in the oxidized state. So after CO binding, a partially reduced, CO-bound cytochrome c oxidase is generated. This is the so-called mixed-valence

Figure V-1: SDS-polyacrylamide gel electrophoresis of cytochrome c oxidase labeled by 1,5-I-AEDANS. The slab gel was photographed with UV illumination to detect the fluorescence of 1,5-I-AEDANS and then stained with Coomassie Blue to locate the subunits as indicated.

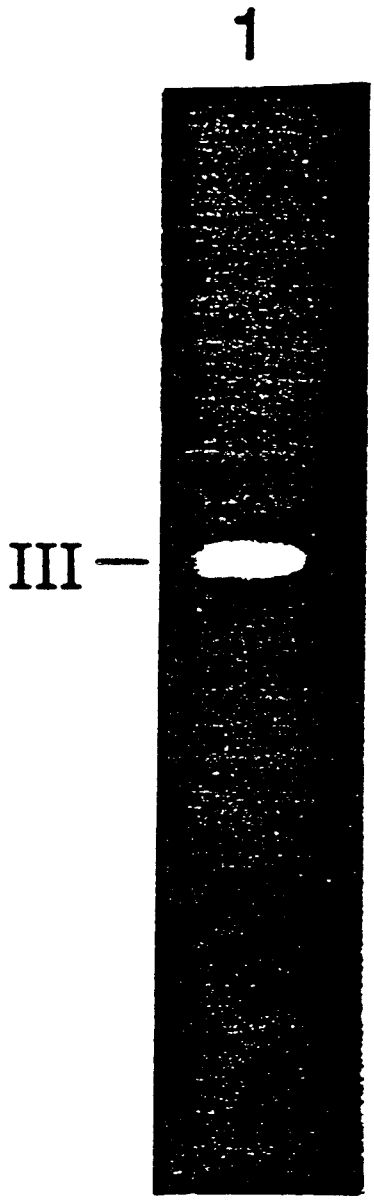
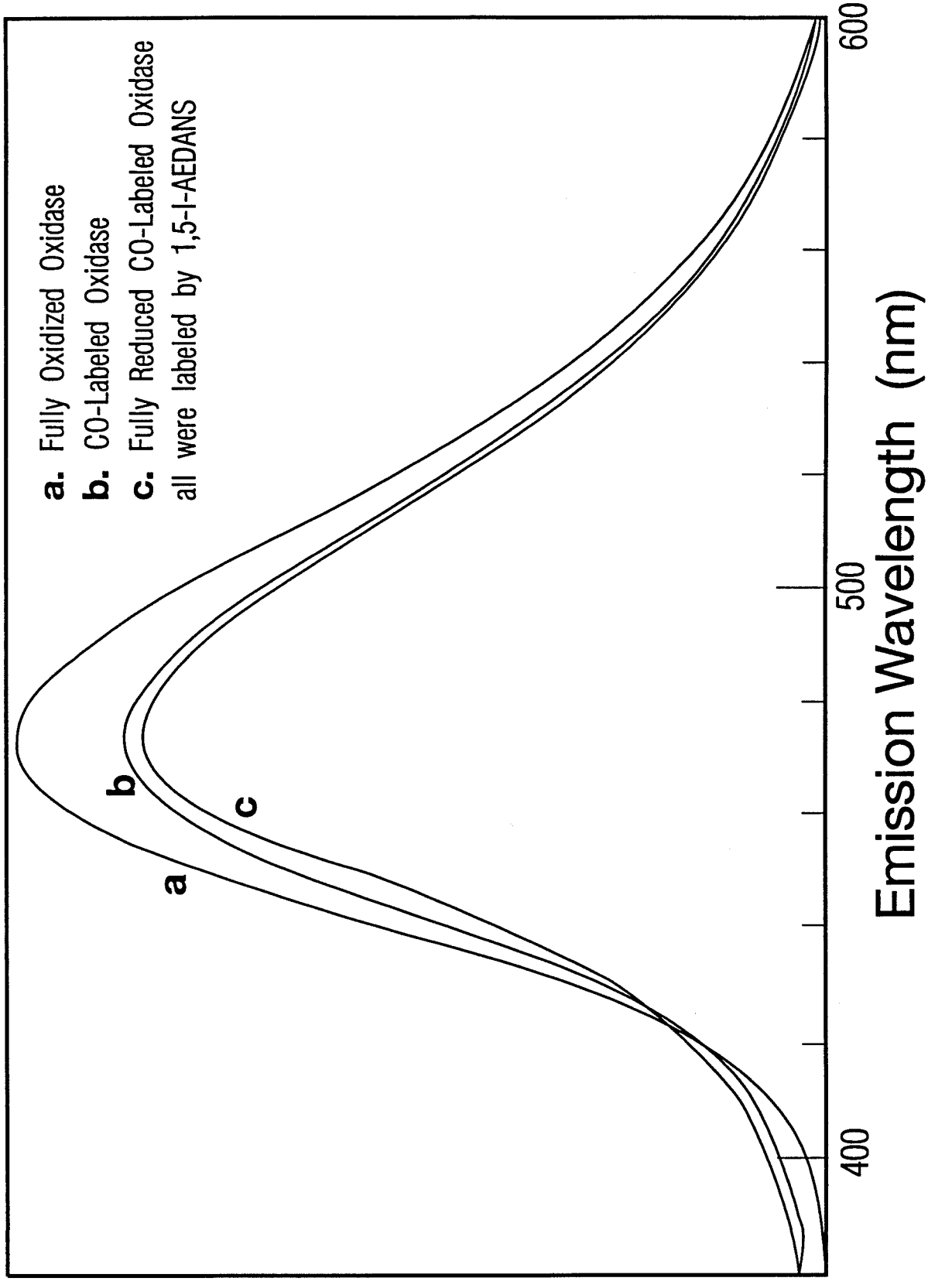

1
III

Figure V-2: Fluorescence spectra of 1,5-I-AEDANS labeled cytochrome c oxidase. (a) resting; (b) CO-bound mixed-valence; and (c) CO-bound fully-reduced enzyme. All emission spectra were obtained using a solution containing 2 μM of the labeled enzyme, 50 mM K-Phos, 0.1% lauryl maltoside at pH 7.4. The excitation wavelength was 340 nm, and the excitation and emission slit widths were 8 nm and 4 nm, respectively.

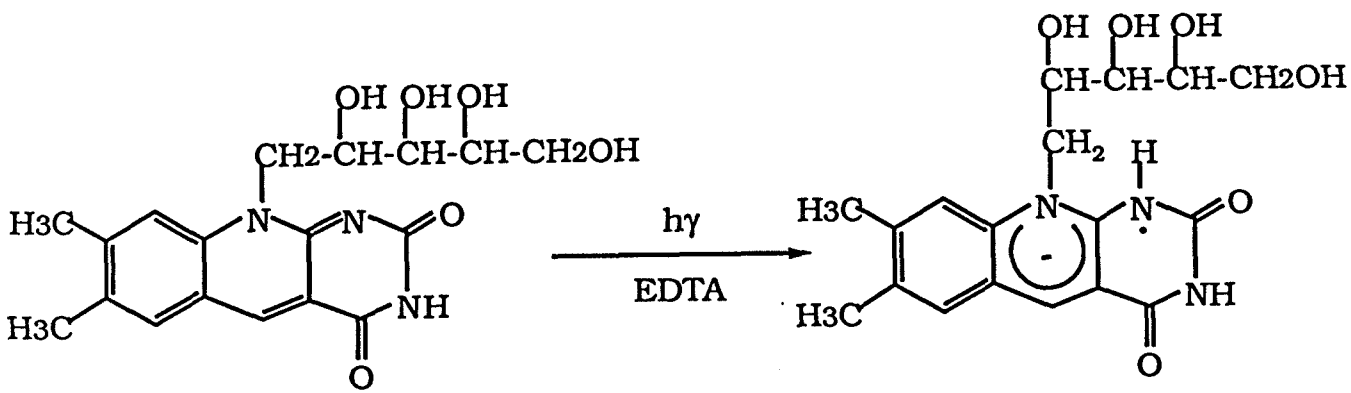

a. Fully Oxidized Oxidase
b. CO-Labeled Oxidase
c. Fully Reduced CO-Labeled Oxidase
all were labeled by 1,5-I-AEDANS
a
b
c
400
500
600
Emission Wavelength (nm)
Fluorescence Intensity
(arbitrary units)

cytochrome c oxidase. With the fluorescence probe, 1,5-I-AEDANS, being specificially labeled on cytochrome c oxidase, the fluorescence spectrum of CO-bound mixed-valence enzyme can be measured as shown in Figure V-2b. Compared with the fluorescence spectra of the resting enzyme, a decrease of the fluorescence intensity is observed upon partially reduction of the enzyme.

The CO-bound mixed-valence cytochrome c oxidase can be converted into a CO-bound, fully reduced state by adding a slight excess amount of sodium dithionite. Since strictly anaerobic condition is required to prepare the CO-bound cytochrome c oxidase, in order to fully reduce the mixed-valence enzyme, only a very small amount of sodium dithionite is needed. As shown in Figure V-2c, a further decrease of the fluorescence intensity is obtained when the partially reduced CO-bound cytochrome c oxidase becomes fully reduced. Although the extent of reduction in intensity is small, this change is always observed.

In another approach, a photoliable system of 5-deazariboflavin (Hazzard et al., 1991) is used to study the redox-linked conformational changes of cytochrome c oxidase. In this experiment, cytochrome c is rapidly reduced by flavin semiquinone generated by flash light excitation of 5-deazariboflavin in the presence of EDTA. Afterwards, ferrocytochrome c transfers electron to cytochrome c oxidase (Scheme I). 5-Deazariboflavin does not absorb significantly in the 480 nm region, which is the wavelength of 1,5-I-AEDANS's emission. As a result, it is a suitable reducing reagent under the current experimental conditions.

Sheme I Mechanism of cytochrome c oxidase reduction by 5-deazariboflavin.



5-DRF **5-DRF•**

(1) **5-DRF• + $(Cyt\ C)_{ox}$ $\xrightarrow{k_{2a}}$ $5\text{-}DRF_{ox}$ + $(Cyt\ C)_{red}$ + H^+**

(2) **5-DRF• + $(CcO)_{ox}$ $\xrightarrow{k_{2b}}$ $5\text{-}DRF_{ox}$ + $(CcO)_{red}$ + H^+**

(3) **$(Cyt\ C)_{red}$ + $(CcO)_{ox}$ $\longrightarrow$ $(Cyt\ C)_{ox}$ + $(CcO)_{red}$**

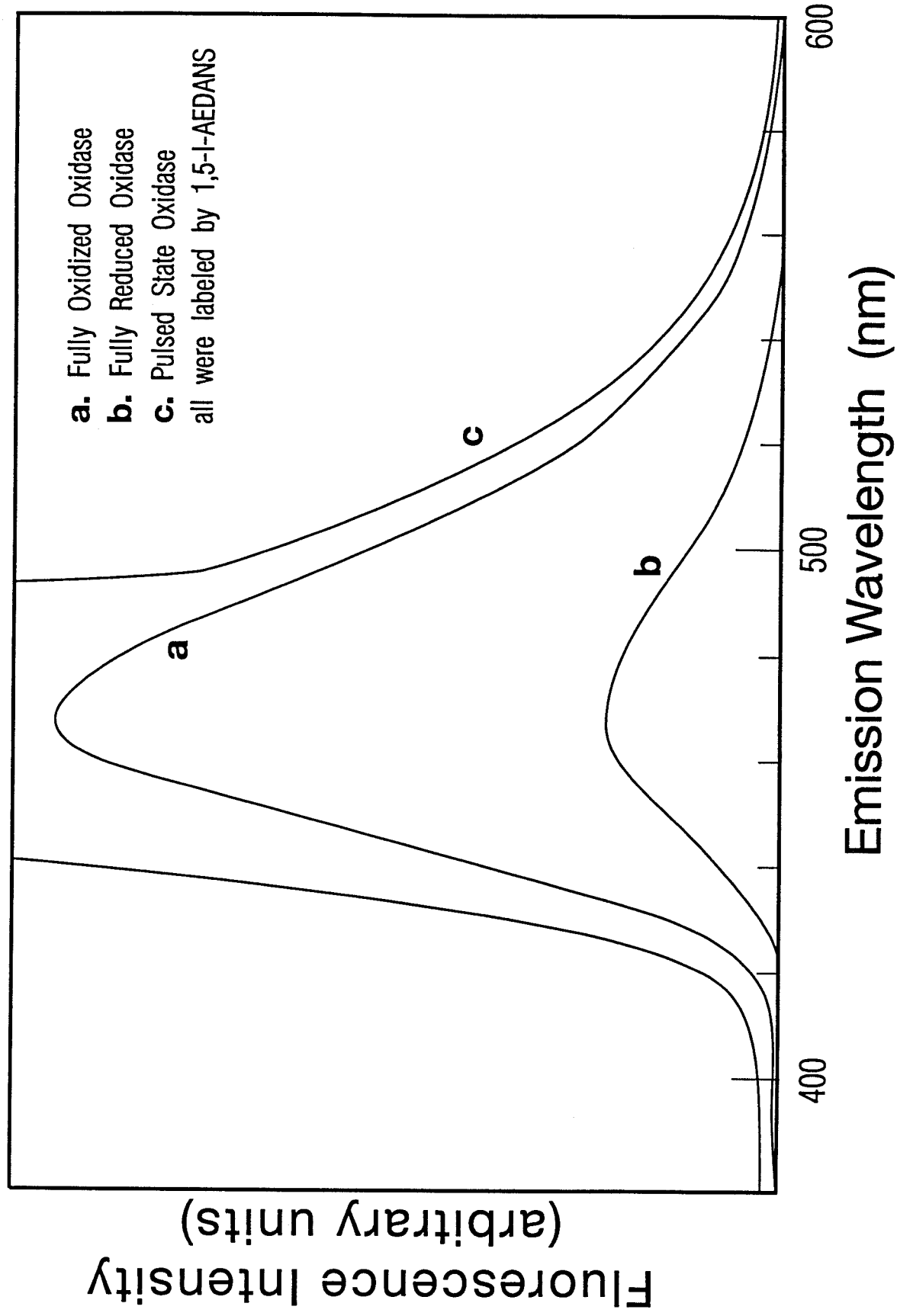
Figure V-3a shows the fluorescence spectrum of a solution contains 5-deazariboflavin, EDTA, cytochrome c, and 1,5-I-AEDANS labeled cytochrome c oxidase. After light irradiation, there is a substantial decrease in the fluorescence intensity with respect to that of the starting system in which cytochrome c oxidase is fully oxidized and resting (Figure V-3b). UV-visible spectrum shows that cytochrome c oxidase is fully reduced immediately after flash light irradiation.

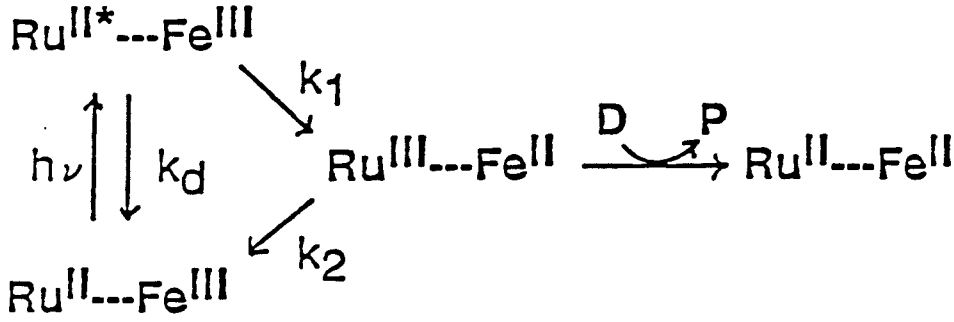
The fluorescence intensity increased significantly, even higher than that of the fully oxidized resting form enzyme after a few bubbles of air were introduced to the reaction mixture by a syringe (Figure V-3c). UV-visible spectrum shows cytochrome c oxidase is converted into the fully oxidized state, namely the pulsed state.

Another photoliable system, ruthenium polypyridine labeled cytochrome c derivative, is also used to investigate the conformational changes of cytochrome c oxidase. This system consists of cytochrome c covalently labeled by $Ru^{II}(bpy)_2(dcbpy)$ at lysine 86 residue and 1,5-I-AEDANS labeled cytochrome c oxidase. The ruthenium polypyridine group can be photoexcited and then transfers an electron to the heme of cytochrome c. EDTA is used as electron quencher to block the ruthenium(III) thermal back reaction (Scheme II). Subsequently the ferrocytochrome c transfers electron to cytochrome c oxidase. The derivatized cytochrome c has an emission centered at 662 nm, without any overlap with the emission of 1,5-I-AEDANS.

Figure V-4a shows the fluorescence spectra of a solution containing ruthenium polypyridine labeled cytochrome c, EDTA, 1,5-I-AEDANS labeled

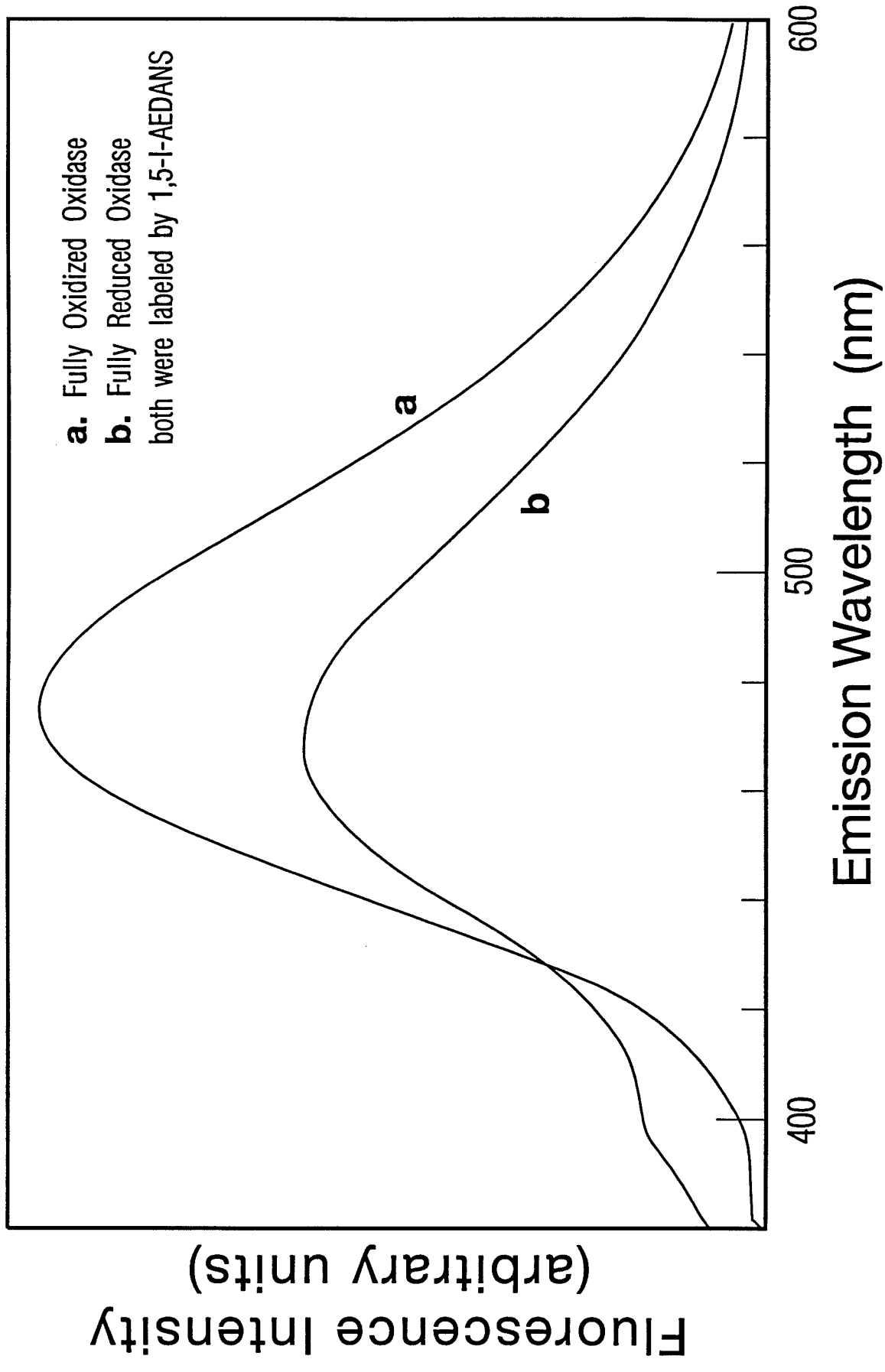
Figure V-3: Effect of oxidation/reduction on the fluorescence of 1,5-I-AEDANS labeled cytochrome c oxidase. The fluorescence emission spectra were recorded with excitation wavelength of 340 nm. Solutions contained 4 μM 1,5-I-AEDANS labeled cytochrome c oxidase, 4 μM cytochrome c, 100 μM 5-deazariboflavin, 1 mM EDTA, 110 mM KCl in 5 mM Tris-Cl, 0.1% lauryl maltoside, pH 7.4 buffer. (a) resting ; (b) fully reduced; (c) pulsed cytochrome c oxidase.


a. Fully Oxidized Oxidase
b. Fully Reduced Oxidase
c. Pulsed State Oxidase
all were labeled by 1,5-I-AEDANS
a
b
c
400
500
600
Emission Wavelength (nm)
Fluorescence Intensity
(arbitrary units)



Scheme II

Figure V-4: Effect of oxidation/reduction on the fluorescence of 1,5-I-AEDANS labeled cytochrome c oxidase. The reaction mixture contained 1.5 μM of 1,5-I-AEDANS labeled cytochrome c oxidase, 1.5 μM $Ru(bpy)_2(dcbpy)$-cytochrome c, 1 mM EDTA, 5 mM Na-Phos, 0.1% lauryl maltoside at pH 7.4. (a) resting form enzyme; (b) fully reduced enzyme


a. Fully Oxidized Oxidase
b. Fully Reduced Oxidase
both were labeled by 1,5-I-AEDANS
a
b
400
500
600
Emission Wavelength (nm)
Fluorescence Intensity
(arbitrary units)

cytochrome c oxidase. Upon light flashes, UV-visible spectra shows both cytochrome c and cytochrome c oxidase are reduced. Concomitant with the redox state changes, a decrease in fluorescence intensity is also observed (Figure V-4b). As a control experiment, the fluorescence emissions of ruthenium complex labeled cytochrome c of both reduced and oxidized states are measured under the same conditions. No emission can be detected (data not shown).

Discussion

The majority of past reports regarding the redox-linked conformational changes of cytochrome c oxidase have been based upon indirect evidence. Spectroscopic studies should be able to provide important and direct information concerning this issue since spectroscopic protocols allow direct monitoring of protein structural changes. Fluorescence has become a powerful tool in biochemistry because of its great sensitivity and its capacity to yield information on molecular association and conformation. Changes in fluorescence spectra are indicative of conformational changes since the micro environment of amino acid residues in the protein matrix is altered (Lackowiz, 1983). So fluorescence spectroscopy can be applied to as one of the few techniques to study conformational changes upon oxidoreduction of cytochrome c oxidase.

Tryptophan fluorescence spectra has been used to probe conformational changes occurred during the turnover of cytochrome c oxidase (Ferreira-Rajabi and Hill, 1989; Smith, unpublished results, Li, unpublished data). However, only a small group of the 52 tryptophan

residues found in cytochrome c oxidase can have their fluorescence spectra been observed. In addition, the maximum emission wavelength of tryptophan (340 nm) is subject to absorbances of other species used in experiments. 1,5-I-AEDANS labeled cytochrome c oxidase provides a valuable alternative for fluorescence studies of conformational changes in the matrix of the enzyme. It is observed that 1,5-I-AEDANS exclusively labels on subunit III of cytochrome c oxidase, presumably Cys-115, since the same cysteine residue has been implicated in labeling experiments by iodoacetamide (Darley-Usmar et al., 1981) and thionitrobenzoate-yeast cytochrome c (Darley-Usmar et al., 1984; Malatesta et al., 1982). There are also other cysteine residues on subunit III and some other subunits, but they are not reactive with 1,5-I-AEDANS (Malatesta et al., 1982; Hall et al., 1988). So the fluorescence spectrum of the singly specifically labeled 1,5-I-AEDANS will not be interrupted by each other as in the case of multiple labeling. Also the 1,5-I-AEDANS labeled cytochrome c oxidase has the same UV-visible and EPR spectral properties as those of the native cytochrome c oxidase, indicating no major changes in protein conformation upon the labeling of 1,5-I-AEDANS.

When 1,5-I-AEDANS labeled cytochrome c oxidase is bound with CO, the enzyme is in a partially reduced state. Adding a bit excess reducing reagent, the enzyme is reduced further into a fully reduced state. Accompanying these redox state changes of cytochrome c oxidase, the fluorescence intensity of the probe labeled on the enzyme decreases. One may argue that the quenched fluorescence is due to energy transfer from the 1,5-I-AEDANS label to some other moieties in cytochrome c oxidase. The UV-visible spectrum of cytochrome c oxidase shows that the enzyme does absorb in the region of 480 nm which is the maximum of AEDANS emission. The

absorption however is quite small, compared to those main absorptions of the enzyme. When cytochrome c oxidase is partially reduced, followed by full reduction, it absorbs less at 480 nm in a manner consistent with the extent of its reduction (Figure V-5). Accordingly, if the fluorescence intensity change upon reduction of cytochrome c oxidase is due to energy transfer, the change of intensity in the region of 480 nm should be in the direction of increase in the extent of reduction of the enzyme. However, the results obtained from experimental measurements are against the view that fluorescence changes observed here are due to energy transfer. The fluorescence intensity changes between CO-bound mixed-valence and CO-bound fully-reduced enzyme is small. But this change is always reproducible. Ferreira-Rajabi and Hill (1989) reported similar pattern of the fluorescence change for the same process although they monitored the tryptophan fluorescence of which the emission maximum is at 328 nm.

As shown in Figure V-6, the photoliable system also absorbs less in the region of 480 nm after light irradiation converting the enzyme in the system from resting form into fully reduced state. It has been shown that 5-deazariboflavin does not absorb in the 480 nm region, and although 5-deazariboflavin absorbs at 337 nm, the absorption at 340 nm remains almost unchanged when the system undergoes oxidoreduction process. The direction of fluorescence change is consistent with the oxidoreduction process in all the experiments. It is very interesting to observe that the fluorescence intensity recovers and even exceeds the original intensity level when the cytochrome c oxidase turns over. Although cytochrome c oxidase returns to the fully oxidized state after reacting with dioxygen, the system gives rise to a significantly different fluorescence spectrum. This however should be

Figure V-5: Absorption spectra of CO-bound mixed-valence cytochrome c oxidase (dotted line) and CO-bound fully-reduced cytochrome c oxidase (solid line).

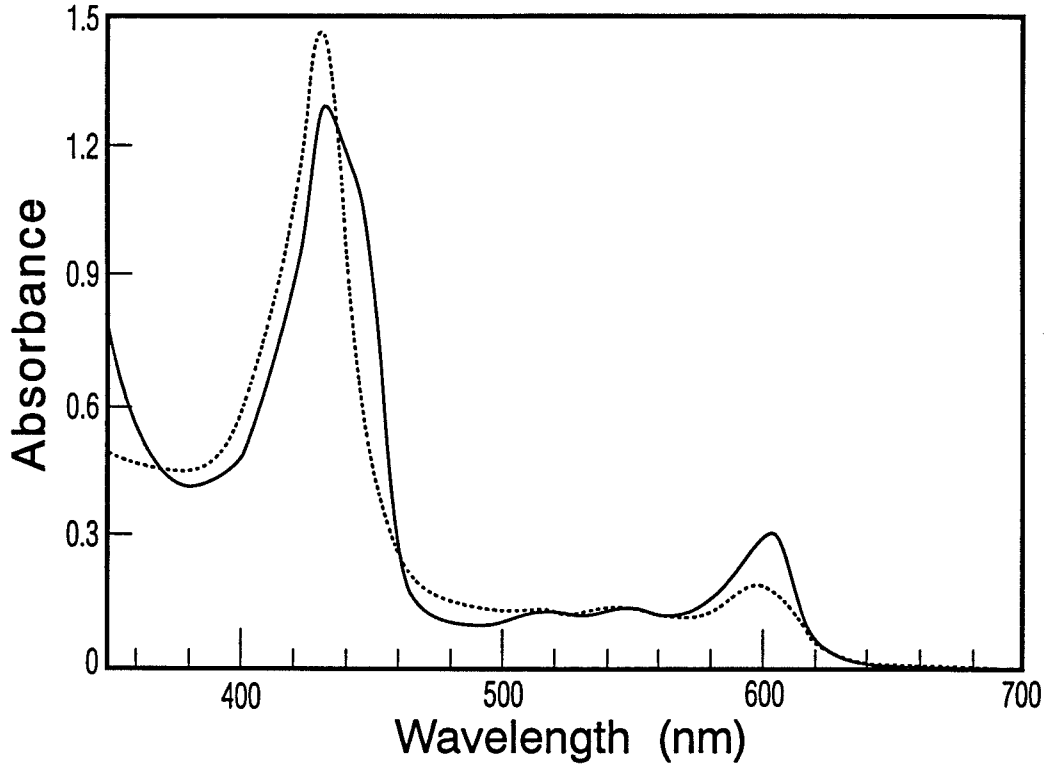
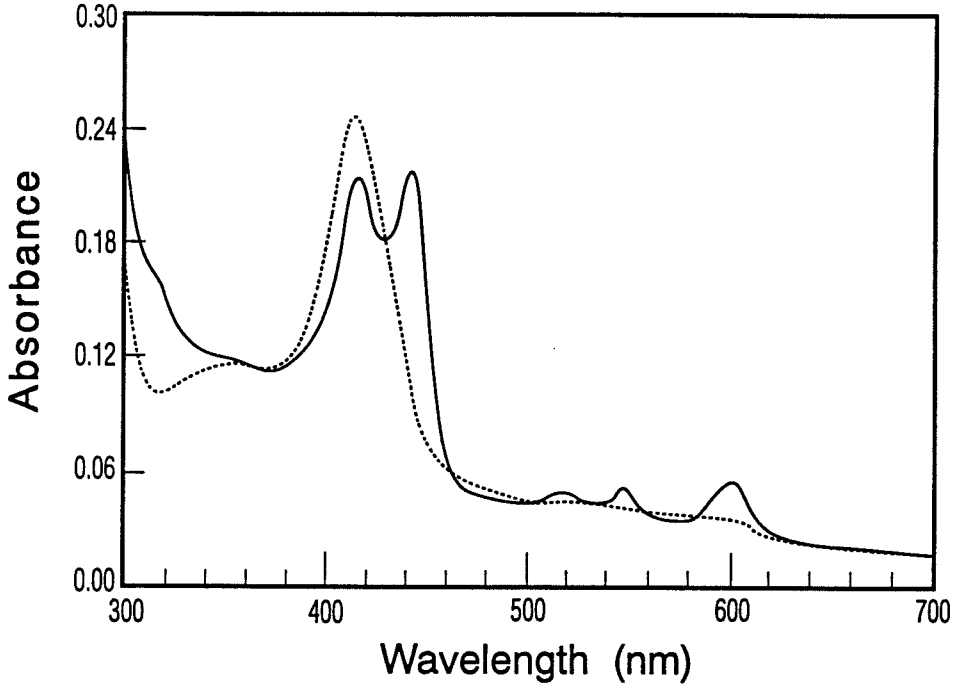

1.5
1.2
0.9
0.6
0.3
0
Absorbance
400
500
600
700
Wavelength (nm)

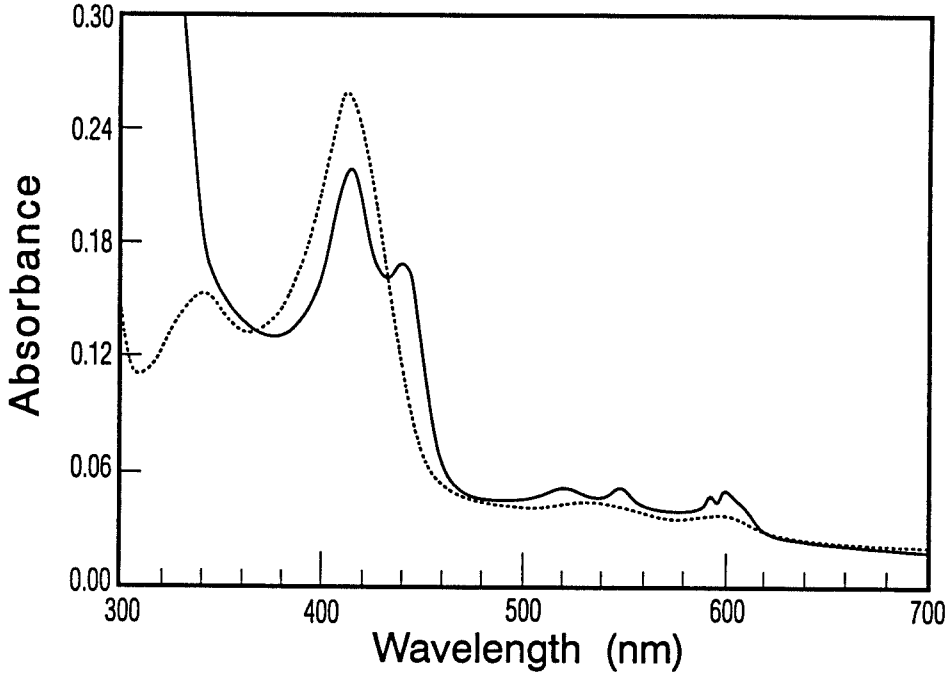
Figure V-6: Absorption spectra of 4 μM 1,5-I-AEDANS labeled cytochrome c oxidase with 4 μM cytochrome c, 100 μM 5-DRF, 1 mM EDTA, 110 mM KCl in resting form (dotted line) and fully-reduced state (solid line). The buffer contains 5 mM Tris-Cl, 0.1% lauryl maltoside, pH 7.4.


0.30
0.24
0.18
0.12
0.06
0.00
Absorbance
300
400
500
600
700
Wavelength (nm)

expected since cytochrome c oxidase is now in the so-called "pulsed state" after turns over, a highly active form of the enzyme. UV-visible spectroscopy may not be able to discern differences between pulsed and resting state of cytochrome c oxidase. Fluorescence spectroscopy, on the other hand, is more sensitive to local and global conformational changes which appear to be the basic difference between these two states of the enzyme. This also explains why fully reduced, CO-bound cytochrome c oxidase and fully reduced cytochrome c oxidase exhibit different extent of decrease in fluorescence intensity (Figure V-2, Figure V-3). It seems that the binding of CO to cytochrome c oxidase locks the enzyme into certain less flexible conformation, resulting in less changes in fluorescence intensity.

The $Ru(bpy)_2(dcbpy)$-lysine group has the same 1+ charge as the native lysine amino group, allowing the cytochrome c derivative to form tight 1:1 complex with cytochrome c oxidase at low ionic strength. In experiments using the ruthenium polypyridine cytochrome c and 1,5-I-AEDANS labeled cytochrome c oxidase system, photoinduced reduction are carried out at low ionic strength. The fluorescence intensity of 1,5-I-AEDANS labeled on cytochrome c oxidase decreases when protein complexes in the system are reduced. The cytochrome c derivative does not absorb appreciably at 340 nm. The UV-visible absorbance of this system are about the same at 340 nm of both the oxidized resting state and reduced form. At 480 nm, the absorbance of the reduced system remains almost unchanged (Figure V-7). The patterns of fluorescence change and UV-visible absorbance change are similar to those of the 5-deazariboflavin system. Thus it appears that the protein complex formation does not affect the conformational change of cytochrome c oxidase

Figure V-7: Absorption spectra of Ru-86-cytochrome c and 1,5-I-AEDANS labeled cytochrome c oxidase in the resting form (dotted line) and fully-reduced state (solid line). The solution contains 1.5 μM Ru-86-cytochrome c, 1.5 μM AEDANS labeled cytochrome c oxidase in 5 mM Na-Phos, 1 mM EDTA, 0.1% lauryl maltoside, pH 7.4 buffer.


0.30
0.24
0.18
0.12
0.06
0.00
Absorbance
300
400
500
600
700
Wavelength (nm)

or the fluorescence probe is not sensitive to the binding of cytochrome c to cytochrome c oxidase.

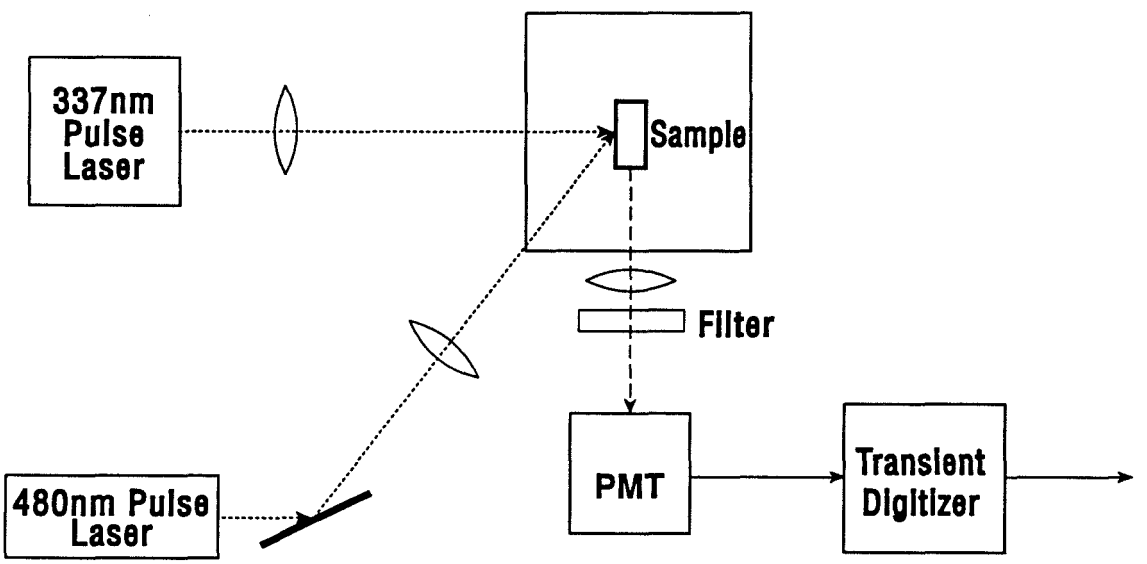
The recognition that cytochrome c oxidase pumps protons during its turnover cycle has led to an intense effort to identify the particular redox center or centers that might serve as a link to couple the free energy available from electron transfer and dioxygen reduction to the proton translocating process for the purpose of ATP synthesis. Many models for redox-linked proton pumping by cytochrome c oxidase rely on redox activity at a single metal center, either cytochrome a or Cu_A, to provide the driving force to pump protons. Several workers have developed a protein conformation dependent model of proton pumping, where they argue in favor of cytochrome a as the site of redox coupling (Wikström, 1978; Artzatbanov et al., 1978; Callahan & Babcock, 1983). The most intriguing experimental evidence in favor of cytochrome a as the site of redox coupling is that the rate of H/D exchange for the protein hydrogen which hydrogen bonds to the cytochrome a formyl oxygen is accelerated by enzyme turnover. An alternative model has been put forth by Chan and co-workers, on the basis of localized conformational transitions in the vicinity of Cu_A site (Gelles et al., 1986). Still some 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. Several experimental results do provide supportive evidence for a role of Cu_A in the proton pumping mechanism. Nilsson et al. (1988) have shown that when Cu_A is modified by *p*-(hydroxymercuri)benzoate, the enzyme no longer sustains proton pumping

when reconstituted into phospholipid vesicles; the vesicles containing the modified enzyme become atypically permeable to protons. Cytochrome c oxidase in which Cu_A has been thermally modified likewise shows this unusual proton permeability when reconstituted into vesicles (Li et al., 1988). These authors argue that the increased proton permeability of the modified Cu_A vesicles is a result from a disruption of the proton gate, creating a passive proton channel through the enzyme.

The data presented here provide clear evidence that there are redox-linked conformational changes in cytochrome c oxidase. At present, our experimental data can not be used to correlate the conformational changes with the catalytic cycle of cytochrome c oxidase. It would be interesting to see if the observed conformational change can be detected in cytochrome c oxidase reconstituted into phospholipid vesicles and whether it would be affected by applied potential gradients across the membrane. It would also be of great interest to observe transient conformational changes during the enzyme's turnover.

We have tried to examine whether electron input from cytochrome c to cytochrome c oxidase causes conformational change in cytochrome c oxidase. The system we used is $Ru(bpy)_2(dcbpy)$ labeled cytochrome c and 1,5-I-AEDANS labeled cytochrome c oxidase. Figure V-8 shows the optical equipment used for the laser-flash fluorescence measurement. Electron transfer was initiated by a 480 nm pulse from an excimer laser. Fluorescence excitation was accomplished with a 337 nm pulse from a nitrogen laser. Pulsed fluorescence excitation gives much better ratio of signal to noise. Upon laser light excitation, the ruthenium complex labeled on cytochrome c

Figure V-8: Block diagram of the apparatus used in the transient fluorescence absorption experiment.


337nm
Pulse
Laser
Sample
Filter
480nm Pulse
Laser
PMT
Transient
Digitizer

transferred an electron to the heme of the protein. Subsequently, ferrocytochrome c transferred the electron to cytochrme c oxidase. Studies have shown that Cu_A in cytochrome c oxidase accepted the electron, then transferred it to cytochrome a. To observe any conformational change involved in the first electron transfer process, attempts were made to detect fluorescence emission at 480 nm, which is the maximum of the 1,5-I-AEDANS emission. It turned out that no fluorescence change was observed. This result suggests that either there is no conformational change during the first electron transfer or our system is not sensitive enough to detect the conformational change associated with this process. According to the model proposed by Chan and co-workers (Chan & Li, 1989), protons are pumped only during the electron transfer steps to the peroxy- and oxyferryl- internediates of the dioxygen reduction cycle. Thus, this model implies that the first two electron transfer steps are non-pumping, while the last two steps are coupled to the translocation of four protons. In this regard, the first electron transfer might not result in any significant conformational change. Hence, the enzyme may remain in one conformation during the first electron transfer. In this conformation, the electron transfer rate is maximized, since the proton pump is effectively disengaged. When dioxygen binds and becomes reduced at the binuclear center, a new conformational state is induced. In this conformation, the intramolecular electron transfer occurs from the pump site to the dioxygen intermediates, thereby engaging the pumping element.

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VI Conclusion

It has been well known that cytochrome c oxidase is an extremely complex protein, both structurally and functionally. The structure of the enzyme has naturally attracted considerable attention. A knowledge of the composition and structure of cytochrome c oxidase is a prerequisite of any attempt toward the elucidation of the functioning mechanism of the protein. An ideal way of collecting the structural information is to obtain the three-dimensional crystal structure. However, it is unlikely that a high resolution structure of the protein will be available in the near future. When isolated from mammalian sources, cytochrome c oxidase is found to be heterogeneous. This heterogeneity makes crystallization difficult to achieve (Capaldi, 1990). However, impressive progress has been made toward obtaining a detailed structure of cytochrome c oxidase. It has been known that cytochrome c oxidase contains a number of metal centers. Those metal centers are perhaps the most intriguing characteristics of the enzyme regarding its composition. Therefore, the characterization of those metal centers have always been the focus of intensive research over several past decades.

The electron transfer mechanism of cytochrome c oxidase must be coordinated with its proton translocation cycle. Accordingly, another major goal of researchers studying cytochrome c oxidase is the elucidation of the chemical mechanism of the redox processes catalyzed by the enzyme. These include the details of the electron input into and the flow of electrons through the enzyme as well as the allosteric interaction involved in the redox processes of the protein. The work presented in this thesis is one of the steps toward understanding these crucial enzymatic processes, hence providing

significant insight into the unique structure and function of cytochrome c oxidase.

In Chapter II, we presented a chemical modification approach to completely remove zinc from cytochrome c oxidase without denaturing the enzyme. This approach has opened up the possibility of studying the nature of zinc in the protein. Our study is consistent with previous EXAFS experiments that at least two cysteine sulfurs are ligated to the zinc ion in cytochrome c oxidase (Scott, 1989). We also concluded that zinc does not play a role in the electron transfer activity of the enzyme.

Chapter III presented the study of electron input into cytochrome c oxidase. The electron input pathway from ferrocytochrome c to cytochrome c oxidase has been extensively studied in recent years. It has been argued that cytochrome a is the primary electron acceptor. However, several recent reports have implicated the central role of Cu_A in the electron input process. Our study shows that when the binding orientation between the two protein complex is altered, the electron input bypasses Cu_A site to cytochrome a directly. Pan et al. (1991) obtained a similar result in which cytochrome a can accept electron directly in the Cu_A-depleted enzyme. Accordingly, we suggest that there may be bi-pathway for the electron input from ferrocytochrome c to cytochrome c oxidase: one via Cu_A, the other via cytochrome a; however the primary input port is via Cu_A.

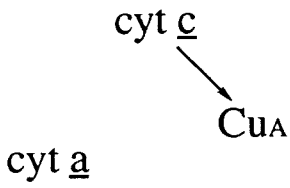
Chapter IV reported a study of intramolecular electron transfer in cytochrome c oxidase following CO photolysis of the CO-bound mixed-valence enzyme. Several works have been reported regarding the issue of

intramolecular electron transfer in cytochrome c oxidase. Unfortunately, this issue is very complex and different studies have resulted in varying findings. We observed that there is a fast electron redistribution from the binuclear metal center to cytochrome a upon CO photodissociation. No further electron transfer to Cu_A was observed.

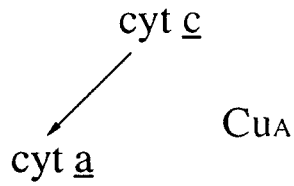
A redox-linked proton pump, like cytochrome c oxidase, must exist in at least two conformations (Blair et al., 1986; Krab and Wikström, 1987). Such conformational changes are necessary because of the requirement of proton and electron gating. Based on this idea, we probed the protein matrix for conformational changes. In Chapter V, we employed a fluorescence probe that identified protein conformational changes associated with the redox-state changes of the enzyme.

The determination of the pathway of initial electron input to cytochrome c oxidase from ferrocytochrome c is of particular interest because of the possible involvement of one of the low potential centers in proton pumping. We would like to combine the data which have been reported with the current results to discuss the possible electron input pathways. Summarizing all the possibilities, Figure VI-1 shows six flow routes of initial electron input pathways. Our study is aimed at identifying which of the six electron transfer pathways is the real case. In the first possible pathway, the electron transfers from ferrocytochrome c to Cu_A site directly and stays at the site prior to the subsequent electron transfer. This pathway obviously contradicts with our observation that cytochrome a is reduced in the electron input process. Actually it is believed that cytochrome a provides the ultimate disposition of the initial electron prior to the subsequent electron

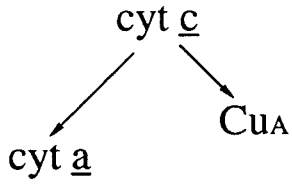
Figure VI-1: Schemes representing the possible electron input pathways from cytochrome c to cytochrome c oxidase.



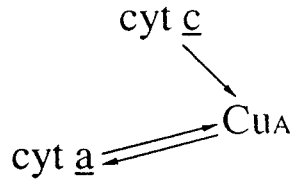
I



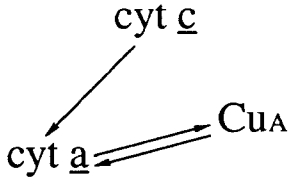
II



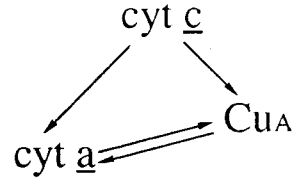
III



IV



V



VI

transport to the dioxygen binding site. The second possible pathway shows that electron transfers to cytochrome a directly and remains at the site for some time. If we only consider the redox potential of cytochrome a (340 mV) is higher than that of Cu_A (280 mV), this pathway might be expected. However, studies from our laboratory have indicated that the reduction of cytochrome a is influenced by the modification at Cu_A site (Pan et al., 1991). Besides, several reports have shown that Cu_A plays an important role in the electron input process (Pan et al., 1991; Pan et al., 1993). Therefore this pathway is not consistent with the experimental evidence. The third pathway displays two distinct electron flow routes from ferrocytochrome c to Cu_A and cytochrome a in parallel, without an electron transfer equilibration between these two metal centers. It should be noted here that the electron transfer process is controlled by kinetic factor, however, the electron equilibration between cytochrome a and Cu_A centers is determined solely on thermodynamics. If this is the case then the reduction of cytochrome a should not be affected by the perturbation at Cu_A site. This is obviously inconsistent with the experimental results from our laboratory (Pan et al., 1991). On the other hand, studies have indicated that there is an electron redistribution from Cu_A to cytochrome a at a rate of $1.8 \times 10^4\ s^{-1}$ (Kobayashi et al., 1989). The fourth possibility hypothesizes that the electron enters Cu_A site first, then the electron passes on to cytochrome a site by electron equilibration. In this case, if Cu_A is removed by chemical modification, the electron transfer would not occur. Because Cu_A is depleted, the protein cannot accept electron from ferrocytochrome c. There is no mention of the subsequent equilibration in this hypothesis. Similarly in experiments using the ruthenium modified cytochrome c at lysine 86 derivative, if the hypothesis is correct, the reduction of cytochrome a would not happen since no reduction of Cu_A was observed.

These extended results are all in conflict with our experimental observations. Accordingly the electron input pathway is not the one described in the fourth hypothesis. The fifth pathway shows that the electron transfers to cytochrome a only, and an electron equilibration exists between cytochrome a and Cu_A to redistribute the electron. Since in this case the Cu_A site eventually receives the electron, an reduction of Cu_A in the input process should be observable. As reported, we did not observe any reduction of Cu_A from studies of electron input from Ru-86-cytochrome c to cytochrome c oxidase. On the other hand, the fifth hypothesis does not agree with the reaction stoichiometry report from this laboratory, in which there is a 1:1 molar ratio of reoxidized ferrocytochrome c to reduced cytochrome a (Pan et al., 1991). Only the sixth proposed pathway can explain all of our experimental observation. In this pathway, the electron can go directly either to Cu_A or to cytochrome a sites. So as the binding orientation is altered and Cu_A site is depleted, electron still can enter cytochrome c oxidase via the cytochrome a site. In the native enzyme, electron transfers to Cu_A directly, then Cu_A redistribute the electron to the cytochrome a site because the cytochrome a site has a higher redox potential (Pan et al., 1991; Pan et al., 1993). Although both cytochrome a and Cu_A centers can accept electron, the experimental evidence strongly implicates that Cu_A plays a predominant role in the initial electron input process. Perhaps Cu_A is the primary electron receptor, accepting an electron directly from ferrocytochrome c and then transferring it to the cytochrome a site. However, we still cannot rule out the possibility that Cu_A is just a regulator, which regulates the rate of direct electron transfer from ferrocytochrome c to cytochrome a.

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