ATP Modulation of the Electron Transfer between Cytochrome c and Cytochrome c Oxidase

Thesis by

Jian Lin

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California 91125

1995

(Submitted November 7, 1994)

Dedicated to my wife, Meijuan Zhang, a very special friend

ACKNOWLEDGMENTS

The first and most important person, to whom I would like to give my thanks, is my graduate advisor Dr. **Sunney Chan**. While I am writing this Ph. D. thesis, I still remember the time when I just started my life here at Caltech, I could hardly speak English correctly and I did not even know how to run a UV-Visible machine. It is Sunney who showed me his care, patience, and gave me the time to adjust myself to the exciting life of Caltech and the Chan group. He helped me to grow to be independent by allowing me to make mistakes and learn from those. He always gave me his valuable advice's and his scientific intuition. Without all the supports from Sunney, the present thesis will be impossible. Thank you Sunney. I will miss you.

I would like to thank the following people who have contributed directly to my graduate research: especially Dr. **Lian-ping Pan**, who helped me to break into the field of cytochrome c oxidase and taught me a lot of experimental techniques, Dr. **Shuguang Wu**, who collaborated with me on projects here at Caltech.

I would also like to thank the following people for contributing their insight into the questions raised by this work: **Siegfried Musser**, **Ted Dimagno**, **John Evans** and **Hoa Nguyen**.

I would also like to thank all of the people who have been part of the Chan Group while I have been at Caltech, especially: **Zhuying Li**, **Randy Larsen**, **Silvia Cavagnero**, **Ruth Ann Bertsch**, **Ron Rock**, **Wa-to Lau**, and **Guangyang Wang**.

Finally, I wish to thank my wife and my mom for their love and support.

ABSTRACT

The mechanism by which ATP modulates the electron transfer activity of cytochrome c and cytochrome c oxidase (CcO) has been extensively investigated by the present thesis. The subunit location of magnesium ion of CcO has been identified as the subunit IV by chemical modification and amino acid sequence analysis. The pathway for the first electron input from cytochrome c to CcO has been studied by laser flash photolysis with native, type I, type II, Cu_A-depleted CcO. The first electron from cytochrome c inputs directly to either heme a or Cu_A with different rate. The electron input to Cu_A was finally deposited to heme a via fast electron transfer from Cu_A to heme a. The effect of 8-azido-ATP-modification on cytochrome c activity was examined by transient electron transfer, steady-state, and binding studies. The 8-azido-ATP-modification does significantly decrease the rate of electron transfer from heme c to heme a of CcO. The retardation effect of the 8-azido-ATP-modification on cytochrome c is largely due to the disruption of the cytochrome c docking surface for CcO, which results in different docking conformation assumed by modified cytochrome c to CcO. On the other hand, the effect of ATP binding to CcO on the electron input from cytochrome c to heme a has been extensively assayed by transient absorption experiments. 8-azido-ATP-modification of CcO has been shown to have similar effect on steady-state kinetics, binding affinity for cytochrome c and first electron input rate from cytochrome c as ATP non-covalent binding. The 8-azido-ATP-modification or ATP non-covalent binding to CcO decreases the electron transfer rate from cytochrome c to heme a to about 60% of that of native enzyme by the perturbation of the cytochrome c binding on CcO, which leads to a change of electronic coupling and/or branching of the electron input from cytochrome c to Cu_A (or heme a). The binding of ATP to CcO have been probed by ATP analog, TNP-ATP. The binding of TNP-ATP to CcO have been shown to have higher affinity than ATP itself. The higher binding affinity of TNP-ATP to CcO is due to a binding energy contributed by the interaction of TNP group with detergent. This high affinity TNP-ATP binding is relevant to the ATP effect as shown by steady-state kinetics, and binding studies. The ATP binding site at subunit IV of CcO is, thus, very close

to lipid bilayer. The magnesium ion, located in subunit IV, is likely to provide the binding site for the ATP binding to CcO.

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Abbreviations

ADP, adenosine 5'-diphosphate

ATP, adenosine 5'-triphosphate

8-azido-ADP, 8-azido-adenosine 5'-diphosphate

8-azido-ATP, 8-azido-adenosine 5'-triphosphate

CcO, cytochrome c oxidase

CcP, cytochrome c peroxidase

DCCD, N, N'-dicyclohexyl carbodiimide

5-DRF, 5-deazariboflavin

EDTA, Ethylenediamine- tetraacetic acid

EGTA, Ethylenebis (oxyethylenenitrilo) tetraacetic acid

Hepes, N-[2-hydroxylethyl] piperazine - N' - [2-ethane sulfonic acid]

NCD-4, N-cyclohexyl-N'-(4-dimethylaminonaphthyl) carbodiimide

SDS, sodium dodecylsulfate

TMPD, N, N, N', N'-tetramethyl-*p*-phenylenediamine

TNP-AMP, 2' (or 3')-O-(2,4,6-trinitrophenyl) adenosine 5'-monophosphate

TNP-ATP, 2' (or 3')-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate

Tris, tris [hydroxymethyl]-aminomethane hydrochloride.

Chapter I

General Introduction

Cytochrome c and cytochrome c oxidase: general background

Oxidative phosphorylation is the process in which ATP is formed as electrons are transferred from NADH or $FADH_2$ to O_2 by a series of electron carriers. It is major ATP source in aerobic organisms. The oxidative-phosphorylation is carried out by respiratory assemblies that are located in the inner mitochondrial membrane. The respiratory chain, consisted of series membrane proteins (called complex I, II, III, IV) and other electron carriers (i. e., ubiquinone, cytochrome c), catalyzes the electron flow from NADH and $FADH_2$ to dioxygen and couples the energy provided by the electron transport to produce electrochemical potential across the inner mitochondrial membrane. Among these proteins, cytochrome c oxidase (complex IV, CcO), the terminal enzyme in the respiratory chain, is the most important enzyme in coupling the redox energy to proton pumping, since the redox energy from its electron donor, cytochrome c, to electron acceptor, dioxygen, is largest (24 Kcal/mol). the electrochemical energy of the proton gradient across the inner mitochondrial membrane was finally utilized by F_0F_1 ATPase as energy source to synthesize ATP from ADP and inorganic phosphate.

Cytochrome c, the electron carrier between cytochrome c reductase and cytochrome c oxidase, is a soluble protein with a prosthetic group called heme c, which functions as the redox center of this protein. The amino acid composition and three-dimensional structure of this protein is well conserved through 1.5 billion years of evolution. 26 among 104 amino acids are conserved throughout eukaryotic organism and the three-dimensional structures of cytochrome c from different sources are extremely similar to each other. This conservation of the structure of cytochrome c allows the cytochrome c from any species to be the substrate of reductase and oxidase from any other species of eukaryotic, albeit the activity and detail kinetics may be somewhat different. Since the size of this protein is relatively small, and it is readily separated from the other proteins of mitochondria, cytochrome c is the most well studied protein in the respiratory chain.

The interaction of cytochrome c with its redox partner, such as cytochrome c reductase and CcO are through the clusters of positively charge lysine side chain around the heme crevice on the surface of the protein. The binding surface of cytochrome c to either cytochrome c reductase or oxidase have been investigated with kinetic examination of singly modified cytochrome c derivatives or by the studies of lysine's shielded in a protein complex. From the studies of the kinetics of CDNP single lysine modified cytochrome c with CcO and cytochrome reductase (Ferguson-Miller *et al.* 1978a, b, Osheroff *et al.*, 1980, Speck*et al.*, 1979; Konig *et al.*, 1980), the docking surface of cytochrome c with that of cytochrome c oxidase and reductase were proposed. Besides the kinetics studies of single lysine modified cytochrome c, the differential chemical modification approach of Rieder and Bosshard (1978, 1980) accomplished the mapping the lysine's of cytochrome c in a single experiment. Cytochrome c, in the presence and absence of a redox partner, is modified with ^{14}C-labelled reagent. The shielding factor of redox partners to individual lysine served as an indication of the relative importance of the lysine side-chain in the docking. Both experiments agree that lysine 87, 13, and 8 are all involved in the docking of cytochrome c to both its reductase and oxidase. In the experiment of Rieder and Bosshard (1978, 1980), it is also demonstrated that lysine 86 is also important to the docking. The results of the docking surface mapping were confirmed recently by crystal structure of a complex of cytochrome c with its another redox partner in bacterial, cytochrome c peroxidase (CcP) (pelletier and Kraut, 1992). It has been shown that all the lysine's, predicted to be important to the docking to cytochrome c reductase and oxidase by the earlier mapping experiments, are involved in the docking of cytochrome c to CcP.

The binding of cytochrome c to CcO have been studied by direct binding studies. It has been shown that cytochrome c at low ionic strength formed tight complex with CcO. Two apparent binding sites for cytochrome c were determined in either mitochondria (Vanderkooi *et al.*, 1973a, b) or CcO (Ferguson-Miller *et al.*, 1976). Among these two apparent binding sites, one is high

affinity and the other is low affinity. The two apparent binding sites were also observed with steady-state kinetics (Ferguson-Miller *et al.*, 1976). The K_d of the higher and low affinity binding sites for cytochrome c at CcO have been determined to be ~10^{-7} M and ~10^{-6} M, respectively. The apparent high affinity binding site have slower turn over activity than that of low affinity binding site. The high apparent affinity binding are more sensitive towards ionic strength than that of low affinity site. As the ionic strength increase to certain value, or more interestingly as ATP concentration increase to mM range, the apparent high affinity binding site disappears (Ferguson-Miller *et al.*, 1976).

Cytochrome c oxidase (CcO) is a membrane protein consisting of multisubunits. For the bovine heart enzyme which is most extensively studied from the mammalian source, it consisted of 13 subunits (Kadenbach *et al.*, 1983). The first three largest subunits (calls subunit I, II, III) of mammalian CcO are coded by mitochondrial DNA and they play the most vital role in the enzyme while other subunits are coded by nuclear DNA (Capaldi, 1985). The nuclear coded subunits possibly play some regulatory roles in the enzyme activity. The three subunits of bacterial CcO are homologous to the mitochondrial coded subunits (I, II, III) of mammalian enzyme. Comparison between CcO of several sources showed that there are six histidine residues in the membrane in subunits I and two histidine and two cysteines in subunit II fully conserved in all the species. These amino acid residues serve as ligands to the metal centers in the enzyme. Subunit III does not contain any redox active metal ion. The molecular weight of monomeric bovine cytochrome c oxidase is 204,000. In mitochondria as well as in reconstituted vesicles, this enzyme, however, exists as dimmers (Capaldi, 1985).

The active centers of CcO are characterized by specific redox behavior linked with its function. There are two heme a moieties, called heme a and heme a_3 and two copper centers, called Cu_A and Cu_B constitute the redox centers of the enzyme. Heme a is a substituted porphyrin complex (called porphyrin a) of iron where the position 8 is occupied by a formyl group and a long isoprenoid

chain is attached at position 2 of the porphyrin ring. Although heme a and heme a_3 contain the same porphyrin ligand, the axial ligands to iron in these two centers are different. Both the heme a and heme a_3 centers reside on subunit I with the porphyrin a plane almost perpendicular to the membrane, Heme a is a low-spin (S=1/2) iron (III) species at the native state with two histidines, H61 and H378 of subunit I axially coordinated to the metal ion. Heme a_3 has one histidine residue (H376 of subunit I) bound axially. Cu_B also resides on subunit I with three histidine residues bound to it (H240, H290, H291). Heme a_3 and Cu_B have been proposed to be bridge by μ-peroxy group (-Fe-O-O-Cu-) in the active enzyme forming the binuclear active center for oxygen binding. Cu_B and heme a_3 are ~4 Å apart from each other, which facilitated the formation of the bridged complex. Cu_A has been shown (Copeland and Chan, 1989) to reside on the non helical part of subunit II and it is ligated to two histidines (H161, G204) and two cysteine (C196, C200) residues of this subunit in the native state. The distance between heme a and Cu_A in the enzyme is ~10 Å while heme a is ~ 16 Å away from heme a_3, apart from these metal active centers, recent studies indicate that there is another copper, call Cu_X, present along with a zinc and a magnesium ion in the enzyme.

CcO has a high-affinity binding site for it redox partner, cytochrome c. Electrostatic interaction between the positively charged lysine residues of cytochrome c and the negatively charged carboxyl group in subunit II of CcO is important in binding of cytochrome c to CcO. However, hydrophobic interaction between residues of two proteins also seems to play a role in the tight binding of cytochrome c to CcO. Cytochrome c binding site in subunit II of CcO is at a distance of ~5 Å from the Cu_A site. Fluorescence energy transfer studies suggested (Alleyne and Wilson, 1987) that the distance between cytochrome c and heme a is 20-25 Å. Recent transient absorption spectroscopic studies (Hill, 1991; Pan *et al.*, 1991) of cytochrome c with CcO showed that Cu_A is the initial electron acceptor on the oxidase from cytochrome c. The electron then could be subsequently transfered to heme a through a electron transfer rate of about $2x10^4$ s^{-1} (Morgan *et al*, 1989; Kobayashi and Hayashi, 1989), since the redox potential of heme a is higher than Cu_A.

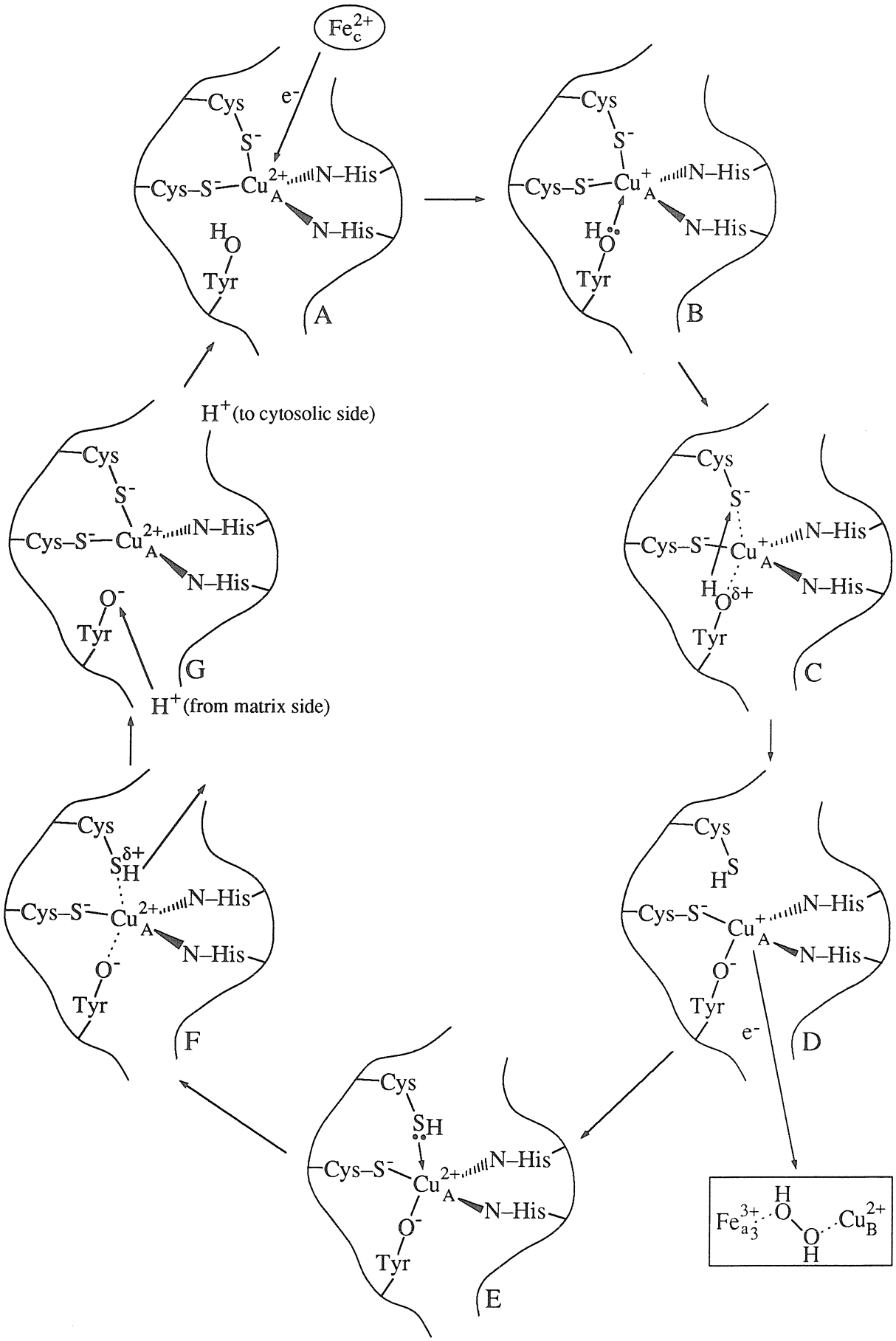
Electron transfer to the heme a site is followed by a series of complex intramolecular electron transfer steps which finally carry the electron to the binuclear Cu_B-heme a3 site. Reduction of oxygen takes place at the binuclear center which involves several transient states such as μ-peroxy (-Fe-O-O-Cu-) and ferryl (-Fe=O) intermediates.

It has been shown that modified bovine CcO which has been specifically changed near the Cu_A site, still shows significant electron transfer activity (Copeland and Chan, 1989; Nilsson *et al.*, 1988; Das and Mazumdar, 1994)). However, such modifications at Cu_A site stop the proton pumping activity of the enzyme. Controlled heat treatment of CcO inactivates the proton pumping property of the enzyme, but this heat treatment species is capable of transferring electron across the membrane. It has been shown that heat treatment modifies the Cu_A site which decouples the proton pumping activity from electron transport property of the enzyme. Heat treatment or partial reduction of the Cu_A site induces the "open" state of the heme a3-Cu_B site. Based on these observations Chan and coworkers proposed that reduction of the Cu_A in CcO causes a ligand exchange by a tyrosine residue for a cysteine group. This change in ligation has been proposed to be linked with the HBC transitions associated with a proton translocation from one residue to the other which provide the mechanism for vectorial uptake of protons from the matrix side and subsequent release of proton to the cytosolic side during enzyme turnover. Figure I.1 shows the Chan model of proton pumping based on the Cu_A site as the redox-proton link.

Biological electron transfer: theoretical and experimental studies

In the respiratory and photosynthetic systems, electrons are transferred between redox centers over large distances, often 15-30 Å. Although the electron donors and acceptors in these reactions are expected to be weakly coupled (non-adiabatic), the electron transfers are remarkably fast and proceed with high specificity. It seems that the electron transfers are not severely hampered by the non-adiabatic.

Figure I.1. The Chan model of proton pumping


Fe_c^{2+}
e⁻
Cys
S⁻
Cys–S⁻
Cu_A^{2+}
N–His
N–His
H
O
Tyr
A
Cu_A^{+}
B
H^+(to cytosolic side)
C
δ+
D
E
F
G
H^+(from matrix side)
Fe_{a3}^{3+}
Cu_B^{2+}

The traditional transition-state description of the rates of chemical reactions involves motion along a potential energy surface in which the reactant atoms gain energy from thermal collisions, surmount an activation energy barrier to achieve a transition state and spontaneously decay into the product. In contrast to these essentially adiabatic reactions, where formation of the transition state leads almost inevitably to the product, the probability of long-distance electron transfer between distant, weakly coupled donors and acceptors from such a transition point is small. Instead, a simple, non-adiabatic description given by Fermi's golden rule is more appropriate.

$$k_{et} = 2\pi/h V_R^2 FC \qquad (1)$$

in which h is the Planck's constant, V_R^2 is the electronic coupling, and FC is the Franck-Condon factor. The rule states that the electron-transfer rate is proportional to the square of the (weak) coupling of the reactant and product electronic states, V_R^2, which in turn is proportional to the overlap of the donor and acceptor electronic wave functions across the space separating electron donor and acceptor molecules.

The nuclear positions of reacting molecules and their environment, so critical to transition state theory, have a central role in the second term of the golden rule. Calculation of this term, described as the density of states by Fermi, is problematic but can be considerably simplified by assuming the nuclei of the donor, acceptor and immediate environment act effectively as harmonic oscillators. In this case, a Franck-Condon-weighted density of states reflecting the overlap of reactant and product oscillators can be written explicitly. The classical view of nuclear motion described by Marcus (Marcus and Sutin, 1985) leads to equation of Marcus classical equation of Franck-Condon factor, whereas a quantum view of the harmonic oscillators leads to the quantum-mechanically correct expressions of Levich and Dogonadze (1959) and of Jortner (1976) and the semiclassical expression of Hopfield (1974).

From analysis of biological electron transfer systems and model proteins, a controversy has arisen concerning the dependence of the electronic coupling on the distance between the donor and the acceptor. It has been suggested both that there is a universal exponential dependence on the distance (Moser *et al.*, 1992) and that the distant dependence is a complex function of the intervening material (Beratan *et al*, 1991; Wuttke *et al.*, 1992). The effective coupling is explicitly dependent on the tunneling energy E, as is clear from the definition for V_R in the one-electron approximation, which is given by

$$V_R = - \sum_{ij} \beta_{Di} G_{ij} (E) \beta_{jA} \qquad (2)$$

$= (H_{bridge} - E)^{-1}$ is the Green's function (Economou, 1990) for the bridge Hamiltonian H_{bridge}. The indices i and j run over all orbitals involved in the transfer process, and β_{Di} (β_{iA}) represents the coupling of ith orbital to donor (acceptor). Evenson and Karplus (1993) have shown that the dependence of electronic coupling to the distance between electron donor and acceptor is sensitive towards the energy difference between the tunneling energy and typical bridge energies. If the energy difference between the tunneling energy and the typical bridge energy is small, the electronic coupling could greatly depend on the intervening materials and it results in non-exponential behavior. If the energy difference between the tunneling energy and the typical bridge energy is large, the electronic coupling becomes insensitive towards the intervening materials, and the electronic coupling will depend exponentially on the donor acceptor distance.

Application of electron transfer theories to an increasing number of biological systems is now possible. This application was restricted to the electron transfer system in which the distance between donor and acceptor is known either from X-ray analysis or, in the case of rigid covalent system, from molecular modeling to an uncertainty of 2 Å. It also required that the electron-transfer rates were measured over a suitable range of free energies measured in the same or similar medium. Recent studies on the bacterial photosynthetic reaction center have provided a particularly

rich source of thermodynamic, kinetic and structural information on intramolecular electron transfer (Marcus and Sutin, 1985; Allen *et al.*, 1987; Chang *et al.*, 1986; Gunner and Dutton, 1989). Another important source of experimental information comes from the electron transfer from ruthenium complex to heme c of the ruthenated cytochrome c (Wuttke *et al.*, 1992).

Intermolecular electron transfer from cytochrome c to CcO and intramolecular electron transfer between metal centers of CcO

Laser-flushed photolysis techniques have been developed to study the electron input from cytochrome c to CcO. To study the electron input from cytochrome c to CcO, a electron donor should be able to donate electron to cytochrome c much faster than the electron input from cytochrome c to CcO when it is flashed with laser pulse. Several electron donor systems have been developed during the years and applied to study the electron transfer between cytochrome c and CcO. Millet and coworkers (Pan *et al.*, 1988, 1990. Durham *et al.*, 1989; Geren *et al.*, 1991; Hahm *et al.*, 1992) have recently developed a new technique to study electron transfer that utilizes cytochrome c covalently labelled with a tris (bipyridine)ruthenium (II) complex. The Ru(II) group can be photoexcited to a metal-to-ligand charge-transfer state, Ru(II*), which is a strong reducing agent, and can transfer an electron to heme c on a nanosecond time scale. Tollin and coworkers (Hazzard *et al.*, 1991) have recently developed another electron injection system, 5-deazariboflavin (5-DRF), for reduction of cytochrome c and apply it to the study of CcO. 5-DRF, upon the photoexcitation, abstract electron from EDTA and become flavin semiquinones. The flavin semiquinones could quickly reduce cytochrome c without any apparent direct reduction of CcO. The reduced cytochrome c could, then, donate electron to CcO and electron input from cytochrome c to CcO could be monitored. Besides the ruthenated cytochrome c and 5-DRF system, Chan and coworker (Larsen *et al.*, 1992) also used uroporphyrin as electron donor to studied electron input to CcO from cytochrome c. Uroporphyrin upon photoexcitation by laser pulse could abstract electron from electron donor, such as NADH, and become reduced. The reduced form of

uroporpherrin could readily donate electron to cytochrome c without apparent direct reduced CcO and the electron input from cytochrome c to CcO could, then, be monitored.

Besides the techniques for studying the electron input from cytochrome c to CcO, other techniques are also used to investigate the intramolecular electron transfer in CcO. These techniques include the flash photolysis of reduced CO inhibited CcO in the presence of O_2 (Hill, 1991), pulse radiolysis (Kobayashi *et al.*, 1989), perturbation method (Morgan *et al.*, 1989), single turn-over experiment (Hill, 1994) and CO photo-dissociate from fully reduced and mixed-valent CcO (Georgiadis *et al.*, 1994).

The electron input from cytochrome c to CcO under different ionic strength was first investigated by Tollin and coworkers (Hazzard *et al.*, 1991) with 5-DRF as electron donor. While the reduction of cytochrome c by flavin semiquinone is independent of ionic strength, the first order rate-limiting one-electron transfer to the heme a showed a marked ionic strength effect, with a maximum rate constant occurring at μ =110 mM (1470 s^{-1}), whereas the rate constant obtained at μ = 10 mM was 630 s^{-1} and at μ = 510 mM was 45 s^{-1}. There was no effect of "pulsing" the enzyme on this rate-limiting one-electron transfer process. These results suggest that there are structural differences in the complex(es) formed between mitochondrial cytochrome c and CcO at very low and more physiologically relevant ionic strength, which lead to differences in electron-transfer rate constants.

Intracomplex electron transfer between ruthenium-cytochrome c derivatives and CcO have also be investigated by Millet and coworkers (Pan *et al.*, 1993). At low ionic strength, all of the derivatives (with ruthenated at different lysine residues) form complexes with CcO. Excitation of Ru(II) to Ru(II*) with a short laser pulse resulted in rapid electron transfer to the ferric heme group of cytochrome c, followed by electron transfer to CcO. The derivatives modified at lysine's 7, 39, 55, and 60 remote from the heme crevice domain showed a fast electron transfer from the heme c to

Cu_A followed by a slower electron transfer from Cu_A to heme a. The rate constant for electron transfer from cytochrome c heme to Cu_A is greater than 10^5 s^{-1}, and the rate constant of the electron transfer from Cu_A to heme a is $2x10^4$ s^{-1}. As the ionic strength increased to physiological value, the complex dissociated, and a rate constant of 2,500 s^{-1} was observed from heme c to Cu_A, while the rate constant from Cu_A to heme a remained unchanged. The electron transfer rate from Cu_A to heme a (20,000 s^{-1}) are agreable from the pulse radiolysis (Kobayashi *et al.*, 1989), flash photolysis (Nilsson, 1992), perturbation method (Morgan *et al.*, 1989).

Besides the first electron input from cytochrome c to CcO and intramolecular electron transfer from Cu_A to heme a which are well studied, some attempts have been made to determine the rate constant of individual steps of the intramolecular electron transfer rate between metal centers of CcO. Einarsdottir and coworkers (Georgiadis *et al.*, 1994) have studied the intramolecular electron transfer and conformational changes in CcO at room temperature following the photo dissociation of CO bound to mixed-valent and fully reduced enzyme. Global analysis of the mixed-valent CO complex transient difference spectra showed the presence of five intermediates with apparent lifetimes of 1.0 μs, 5.2μs, 83.7 μs, 10.5 ms, and 25.3 ms. From the data fitting of the mixed-valent CO bound CcO, electron exchange rate between heme a and heme a_3 and that between heme a and Cu_A could be estimated for the second electron. The rate constant of electron transfer from heme a to heme a_3 and that from heme a_3 to heme a are $1.7x10^5$ s^{-1} and $1.7x10^4$ s^{-1}, respectively, while electron transfer from Cu_A to heme a and that from heme a to Cu_A is $1.2x10^4$ s^{-1} and $6.5x10^3$ s^{-1}, respectively. More intracomplex electron transfer rates have been estimated from the studies of single-turnover of reduced cytochrome c with O_2 (Hill, 1994). By fitting time courses for the reaction intermediates populated during the single turnover of the fully reduced cytochrome c-CcO complex with the reaction scheme of sequential electron transfer steps. Seven rate constant of forward reaction and that of six reversed electron transfer, which represent the third and four electron exchange between four metal centers, have been determined. More importantly, all the forward electron transfer rates are faster than the electron input from cytochrome c to heme a at

physiological ionic strength (slowest among these rate constants is 5 $x10^3$ s^{-1}), except one rate constant which is present in the fourth electron transfer from heme a to binuclear center (800 s^{-1}). Although these experiments could provide some information about the intramolecular electron transfer rate constants, care should be taken to interpret the data, since the involvement of a large amount of variables and assumption about the reaction scheme could introduce great uncertainty in the data fitting.

Regulation of cytochrome c and CcO activity by nucleotides

Based on the observation of the molecular structure of CcO from different organisms, the occurrence of multiple, tissue-specific forms of mammalian CcO, and the variable catalytic activity of isolated CcO, Kadenbach (Kadenbach, 1986) proposed that the nuclear-encoded subunits of CcO function as the regulation factor for controlling the rate of respiratory and the degree of coupling. The presence of the unclear-encoded subunits, which are not directly involved in electron transfer or proton pumping and could differ greatly from tissue to tissue or even in the same tissue, are proposed to provide CcO with specific binding sites for metabolites, hormones, second messengers, and other intracellular signals. These subunits are suggested to function by transmitting the signal of allosteric ligands to the catalytic center (or subunits) of the complex via conformational change. Therefore, a tight association of the regulatory with the catalytic subunits is essential.

The observation that ATP, in its physiological concentration, could abolish the high affinity phase and reduce the activity of low affinity phase in the steady-state kinetics of cytochrome c and CcO (Ferguson-Miller *et al.*, 1976), has led to proposition that ATP function as a regulation factor for controlling the cytochrome c and CcO activity. The physiological meaning of this regulation is obvious. Low ATP concentration in the mitochondria will result in faster respiratory and coupled by higher rate of ATP synthesis and high ATP concentration will slow down the respiratory and

low ATP synthesis rate. By this means, higher organism could regulate the output of ATP according to the demand of the cell metabolism.

In recent years, several studies have contributed to the knowledge of the possible role of ATP in the regulation of the CcO activity *in vivo*. Montecucco *et al.*, (1986) have observed labeling of subunit IV and VII upon photocrosslinking of CcO with a ATP analog, 8-azido-[γ-^{32}P]ATP. It is suggested that subunit IV and one of subunit VII is the binding subunit for ATP. From the results of metal contents of the certain subunits-removed CcO, it is proposed that Mg^{2+} is located in subunit IV and function as the magnesium binding site (Yewey and Caughey, 1987). This binding appears to cause a long-range conformational change in the structure of CcO, which resulted in the change of the conformation of subunit II and consequently the affinity of subunit II for cytochrome c binding (Bisson, *et al*, 1987). It is suggested that the density of the negatively charge phosphate groups present in ATP is the governing factor more than the aromatic ring linked to the sugar moiety, because UTP has the similar kinetic effect to CcO as ATP (Bisson, *et al.*, 1987). The specific kinetic effect of ATP, free from unspecific ionic effect, has been observed by using 8-azido-ATP covalently modified CcO for the kinetic studies, which show similar kinetic effect as free ATP (Huther and Kadenbach, 1986). Extraliposomal ATP increases the K_m for cytochrome c similar to intraliposomal ATP, when ATP effect is observed with reconstituted CcO vesicle (Huther and Kadenbach, 1987), although these two ATP could be greatly different. The interaction of ATP with CcO in the cytosolic side is in an unspecific, solely electrostatic and non-saturatable manner. The interaction of ADP and ATP with CcO from the matrix side show totally different behavior and are more specific. The similar kinetic effect of ATP in the cytosolic side of the reconstitute CcO is observed from both *P. denitrificans* and bovine heart (Huther and Kadenbach, 1988). It is clear that the interaction of the nucleotides in the cytosolic side is not involved in the nuclear DNA encoded subunit(s), because CcO from *P. denitrificans* totally lacks all of them. No influence of nucleotides in the matrix side was observed with the *Paracoccus* enzyme (Huther and Kadenbach, 1988). The binding between ATP and CcO has been studied by Reimann and

Kadenbach (1992), using a ATP analog, TNP-ATP. A stoichiometry of two TNP-ATP per monomeric CcO was determined and a K_d value of 1.6 μM is obtained from titration of CcO with TNP-ATP.

On the other hand, Wallace and coworkers has proposed a regulatory role of ATP binding to cytochrome c at a site near Arg 91 of mammalian enzyme, which is conserved among all cytochrome c known. It is suggested that the ATP effect observed could be due to the binding of ATP to cytochrome c rather than to CcO (Craig and Wallace, 1993). In support of this proposal, Craig and Wallace covalently modified cytochrome c with 8-azido ATP and other ATP analogs, and demonstrate that the modified cytochrome c had reduced ability to restore the oxygen consumption of the cytochrome c depleted mitochondria.

Aim of my doctoral thesis researches

In order to elucidate the mechanism by which ATP regulates the activity of the cytochrome c-oxidase and the detail of the interaction of ATP with cytochrome c and CcO, I carried out several research projects, as an attempt to identify the subunit location of the magnesium which was proposed to be located in subunit IV and function as the ATP binding site, In chapter II, I have developed a method to selectively deplete magnesium from CcO. Following the magnesium depletion, CcO was modified by carboxyl (the putative ligand of magnesium) selective reagent (NCD-4). Comparison between the modification of each subunit by the carboxyl reagent for magnesium depleted CcO and native CcO could reveal the binding subunit of magnesium. Once the subunit for magnesium binding is identified from chemical modification, further sequence analysis of these subunits will provide us with further support for our conclusion.

The understanding of the electron input from cytochrome c to CcO is essential to our knowledge of how ATP regulated the activity of cytochrome c-CcO, since the regulatory effect is exerted through

interfering the interaction between cytochrome c and CcO from earlier studies. In chapter III, we have attempted to investigate how electron input from heme c to heme a or Cu_A of CcO. In order to determine the importance of the Cu_A in the electron input from cytochrome c, Cu_A modified, Cu_A depleted together with the native CcO were used to study the electron input from cytochrome c with flash photolysis method with 5-DRF as electron donor. The ratio of the heme c reoxidation to the heme a reduction will help us in determination of the stoichiometry of the distribution of the first electron on heme a and Cu_A, after input from cytochrome c.

The ATP binding site at cytochrome c and the possible regulatory role of the ATP binding at this site on the activity of the cytochrome c could be a significant contributing factor to what we observed on the ATP regulation of cytochrome c-CcO activity. Understanding of the relation of 8-azido ATP modified cytochrome c to the non-covalent binding of ATP to cytochrome c is very important, as 8-azido ATP modification had significant effect on the cytochrome c activity (Craig and Wallace, 1993). We, therefore in chapter IV, made an attempt to study the detailed mechanism of the reduced activity of the 8-azido ATP modified cytochrome c. To do this, we modified cytochrome c with 8-azido ATP and studied the electron input rate from these modified cytochrome c to CcO with flash photolysis methods with 5-DRF as electron donor. Redox potential of the modification enzyme was also measured to determine the possible effect of the modification on the redox potential of heme c. Binding between native and modified cytochrome c was investigated to assay the effect of modification on the docking of the complex. Effect of free ATP binding to cytochrome c on the binding between cytochrome c and CcO was also determined to compare the modification to the non-covalent binding ATP effect.

As supported by most of the experimental evidences and research groups, ATP interaction with CcO, which results in the change of binding affinity of CcO to cytochrome c and the electron transfer activity. It is important to understand how ATP binding to CcO could affect the electron input from cytochrome c, which is the subject of Chapter V. In order to avoid the problem of ionic

strength and ATP interaction with cytochrome c, CcO was covalently modified with 8-azido ATP. The native and 8-azido ATP modified CcO were then used to study the electron input from cytochrome c by flash photolysis method with 5-DRF. Control experiments on 8-azido ADP modified CcO and native CcO with free ATP at the saturating concentration present in the buffer were performed for comparison. The effect of ATP on the redox centers, especially heme a and Cu_A, were also studied by measuring the redox potentials and by EPR. The binding constants between native or 8-azido ATP modified CcO with cytochrome c were determined to assay the effect of the modification on the binding of the complex.

The details of the binding of ATP to CcO and the properties of the ATP binding site largely remain unknown. In Chapter VI, the binding between ATP and CcO was probed with TNP-ATP using fluorescence techniques (titration, temperature dependent, etc.), steady-state kinetics, direct binding measurement, and also photoaffinity labelling with 8-azido [γ-^{32}P] ATP. The binding parameters will be determined from these experiments. Several important points about the ATP regulatory role of the cytochrome c and CcO will be addressed from the specific binding property of TNP-ATP to detergent micelles. The ATP binding site properties will be extensively investigated in this chapter.

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Chapter II

The Subunit Location of Magnesium in Cytochrome c Oxidase

ABSTRACT:

The magnesium ion in bovine heart cytochrome c oxidase can be depleted up to 75% by heat treatment of the enzyme at 43°C and then dialysis against EDTA buffer solution. The magnesium-depleted enzyme so obtained retains 40% of the activity of the native enzyme. This is the first attempt to deplete magnesium ion from bovine heart cytochrome c oxidase without denaturation of the protein. Magnesium depletion exposes at least one carboxyl group on subunit IV for labeling by N-cyclohexyl-N'-(4-dimethylaminonaphthyl) carbodiimide (NCD-4). The NCD-4 labeling of subunit IV of the magnesium-depleted enzyme is significantly enhanced relative to what is observed for the native and heat-treated oxidase, suggesting that the magnesium ion is located in subunit IV with at least one carboxyl ligand. By comparing the activity of the magnesium-depleted enzyme with that of a control sample of heat-treated oxidase, the influence of divalent magnesium on the activity of the enzyme is assessed.

INTRODUCTION:

Cytochrome *c* oxidase (CcO), the terminal component of the mitochondrial respiratory chain, catalyzes the transfer of electrons from cytochrome *c* to dioxygen, and couples the electron transfer to the active transport of protons across the mitochondrial inner membrane.

CcO is a metalloprotein with a number of metallic cofactors. For the mammalian enzyme, two irons (heme *a* and heme a_3), and two coppers (Cu_A and Cu_B) form the redox-active centers of each CcO monomer (Wikstrom *et al.*, 1981). One zinc and one magnesium are also associated with the monomeric enzyme complex. In addition, another copper called Cu_x, has been implicated with the dimer (Pan *et al.*, 1991 a), the form of the enzyme in the membrane. The mammalian enzyme consists of 13 subunits per monomer (Downer *et al.*, 1976; Kadenbach *et al.*, 1983). The three largest subunits are encoded by mitochondrial genes and are responsible for the electron transfer and proton pumping activities of the enzyme. Heme *a*, heme a_3 and Cu_B are located in subunit I (Winter *et al.*, 1980; Ludwig *et al.*, 1980). Cu_A is located in subunit II (Martin *et al.*, 1988; Hall *et al.*, 1988), which appears to be the binding site of cytochrome *c*. The location of zinc in CcO has recently been shown to be subunits VIa and VIb (Pan *et al.*, 1991 b). At this time, the subunit location of the magnesium and the role of this cofactor in the structure and function of CcO are unknown.

There are two reasons for the paucity of data on the magnesium in CcO. First, the magnesium does not exhibit a spectroscopic signature. Second, the magnesium seems to bind tightly to CcO. An early study by Yewey *et al.* (Yewey *et al.*, 1987) excluded subunits III, Va, VIa and VII as the location of the magnesium ion, since the removal of these subunits by treatment of the enzyme with 1.0% Triton X-100 at pH 9.5 followed by anion-exchange chromatography did not affect the metal stoichiometry of the oxidase. Thus only subunits I, II, IV, Vb, VIb and VIc are possible binding sites of the magnesium ion. Based on the metal assays of Steffens *et al.* (Steffens *et al.*,

1987), the two-subunit CcO from *Paracoccus denitrificans* with only subunits I and II, lacks magnesium. Accordingly, it is very unlikely that the magnesium is associated with subunits I and II. Although these workers proposed that the magnesium ion is located in subunit IV and that it functions as the ATP binding site, following an earlier observation of Montecucco *et al.* (Montecucco *et al.*, 1986) that 8-azido-ATP labels specifically subunits IV and VII, there has not been direct evidence for this proposal.

In this study, we have developed a method to deplete the magnesium from CcO. The putative carboxyl ligand(s) exposed on subunit IV following the magnesium depletion has been labeled by the carboxyl-specific reagent N-cyclohexyl-N'-(4-dimethylamino -naphthyl) carbodiimide (NCD-4). These experiments provide strong evidence that the magnesium ion is located in subunit IV. Finally, we have compared the primary sequences of subunit IV of CcO from several mammalian sources as well as with the sequences of subunit V from *Saccharomyces cerevisiae* and *Neurospora crassa* CcO, the counterparts to mammalian subunit IV in the latter lower eucaryotes (Power *et al.*, 1984). Sequence alignment of these peptides suggests four highly conserved carboxyl-containing amino acids (Glu and Asp) in these peptides. Further comparison of the hydropathy properties of the domains containing these conservative Glu and Asp has led to tentative assignment of at least one of the ligands of the magnesium ion. This prediction appears to be confirmed by the recent report of a conservative carboxyl amino acid in subunit VI of Slime mold (*Dictyostelium discoideum*) CcO. On the basis of sequence comparison, we surmise that Glu/Asp 136 in subunit IV of mammalian enzyme is the most likely candidate to be a ligand of the magnesium ion.

MATERIALS AND METHODS:

Materials—Cytochrome *c* oxidase was isolated and purified from bovine heart mitochondria by the method of Hartzell & Beinert (Hartzell *et al.*, 1974). The enzyme preparation was stored at -80°C

before use. Enzyme concentrations were determined from $\Delta A_{red\text{-}ox}$ at 605 nm using an extinction coefficient of 24 $mM^{-1}cm^{-1}$ (Van Gelder, 1966). NCD-4 was obtained from Molecular Probe (Eugene, OR 97402). Other reagents were of the highest grade available.

Magnesium depletion—Magnesium depletion was obtained by heat treatment of CcO (10 μM CcO in 25 mM phosphate, 0.5 % Tween-80, buffer pH 7.5) at 43°C for 1 hr. The sample was then extensively dialyzed against 25 mM phosphate, 0.2% Tween-80, buffer pH 7.5 and 25 mM EDTA for 12 hr, and again against the same buffer but without EDTA for another 24 hr at 4°C. As a control, another sample was similarly heat-treated, but, in place of the subsequent dialysis steps, the control sample was incubated at 4°C for the same length of time as the magnesium-depleted sample.

Metal ion analyses—Metal assays were performed by direct current plasma emission spectroscopy on a Beckman Instruments SpectraSpan VB Emission Spectrometer. The metal ion concentrations of the sample were determined relative to standard solutions. Two determinations were made for each sample, and the results were averaged. The spectrometer readings were also corrected for trace ions in the final dialysis buffer, which was taken to be the blank control.

Enzyme activity—The activities of the native, heat-treated and magnesium-depleted proteins were determined spectrophotometrically on a HP 8452A diode array spectrophotometer. Assays were performed by following the oxidation of 20 μM ferrocytochrome *c* at 550nm in 50mM sodium phosphate, 0.1% lauryl maltoside, pH 6.0. The concentration of the enzyme was 2-4 nM. The turnover number of the native enzyme was typically about 300 s^{-1}.

NCD-4 modification—NCD-4 was added from a stock solution (100 mM in DMF) to a final concentration of 1 mM to 10 μM CcO (native, heat-treated or magnesium-depleted) dissolved in a buffer of 25 mM phosphate, 0.15% Tween 80, pH 7.5. The reaction was run at 4°C for 12 hr.

The sample was then layered onto 10% (w/v) sucrose, 20 mM phosphate buffer, pH 7.6, centrifuged at 250,000 g for 5 hr, and the pellets were collected for analysis by electrophoresis.

Electrophoresis—The pellets obtained above were dissociated into subunits for 1 hr at 25°C in 8 M urea, 5% SDS. Slab gels were run on a LKB 2001 vertical electrophoresis unit as described by Darley-Usmar *et al.* (Darley-Usmar *et al.*, 1984) using a 7% polyacrylamide stacking gel and a 14% running gel, both containing 6 M urea. The gels of the NCD-4-labeled oxidase were illuminated with UV light and photographed to observe the fluorescence from the N-acylurea. The NCD-4-labeled oxidase gels were subsequently stained with Coomassie Blue.

Sequence analysis—All peptide sequences of subunit IV (mammalian CcO) and subunit V (*Neurospora crassa* and *Saccharomyces cerevisiae* CcO) were obtained from the NIH data bank. Sequences of these peptides were retrieved from the National Center of Biotechnology Information (NCBI) using the Retrieve Network Service. The sequence alignments were also performed at the NCBI using the Blast Network Service. The hydropathy analysis of these sequences was carried out with the PeptideStructure program on the Sequence Analysis System (Seqvax) in the Sequence Analysis Laboratory of the Biology Division at Caltech. The hydropathy analysis is done according to the Kyte-Doolittle method using an averaging window of nine amino acid residues.

RESULTS AND DISCUSSION:

Magnesium depletion—In the present study, we have attempted to remove the tightly bound magnesium ion from CcO. We have shown that with (a) heat-treatment of bovine CcO for 1 hr at 43°C, (b) followed by dialysis in the presence of EDTA for 12hr at 4°C, and (c) further dialysis in the absence of EDTA for 24 hr at 4°C, it is possible to remove the magnesium from the protein. Under these conditions, metal ion analysis (Table 1) indicates that the magnesium has been

TABLE I

Metal contents of magnesium-depleted cytochrome c oxidase

Enzyme preparation	Metal atom ratio			Assignment (per dimer)						
	Cu/Fe	Zn/Fe	Mg/Fe	Fe_a	Fe_{a3}	Cu_A	Cu_B	Cu_X	Zn	Mg
Native oxidase	1.27±0.04	0.47± 0.03	0.56± 0.04	2	2	2	2	1	2	2
Mg^{+2}-depleted oxidase	1.26±0.03	0.42± 0.02	0.12± 0.01	2	2	2	2	1	2	0.5

depleted from the CcO up to 75%. This represents the first successful attempt to deplete the magnesium from CcO.

Activity assay—In Table 2, we have compared the activity of the enzyme, as assayed by ferrocytochrome *c* oxidation, for the native oxidase, the heat-treated enzyme and the magnesium-depleted preparation obtained in this study. The activity of the heat-treated enzyme (75%) is essentially identical to that of the type 1 Cu_A oxidase (80%) reported earlier (Pan *et al.*, 1991 a). Considering that the temperature of the heat-treatment (43°C) in the present study is somewhat higher than that employed to obtain the type 1 Cu_A oxidase (40°C), and that the heat-treated sample has been further subjected to prolonged incubation (36 hr) at 4°C, the comparable activities observed among the various preparations are perhaps surprising. The activity of the magnesium-depleted oxidase is reduced only by 50% relative to that of the heat-treated control. This loss of activity is small.

Labeling of cytochrome c oxidase by NCD-4—The ligand structure of the magnesium in CcO is not known. However, we expect the interaction between the magnesium and CcO to be principally electrostatic in nature. Accordingly, the most reasonable ligand candidates are those amino acid residues with a carboxyl side chain, such as glutamic acid or aspartic acid. If we assume that there is at least one such carboxyl ligand at the magnesium site, and that this carboxyl ligand becomes exposed upon dissociation of the magnesium, then we surmise that this carboxyl ligand could become available for chemical modification. With this rationale, we proceeded to compare the chemical modification by carboxyl specific reagents of the native, heat-treated and magnesium-depleted enzymes. The carbodiimide, NCD-4, has been chosen for this study. The reactivity of NCD-4 toward a carboxyl ligand is similar to that of N, N'-dicyclohexyl carbodiimide (DCCD). The latter has been widely used as a carboxyl modifying and labeling reagent in CcO. Prochaska *et al.* (Prochaska *et al.*, 1981) have studied the DCCD labeling of subunit IV in native bovine heart CcO and have showed that the labeling occurs on the CNBr fragment between methionine 86 to

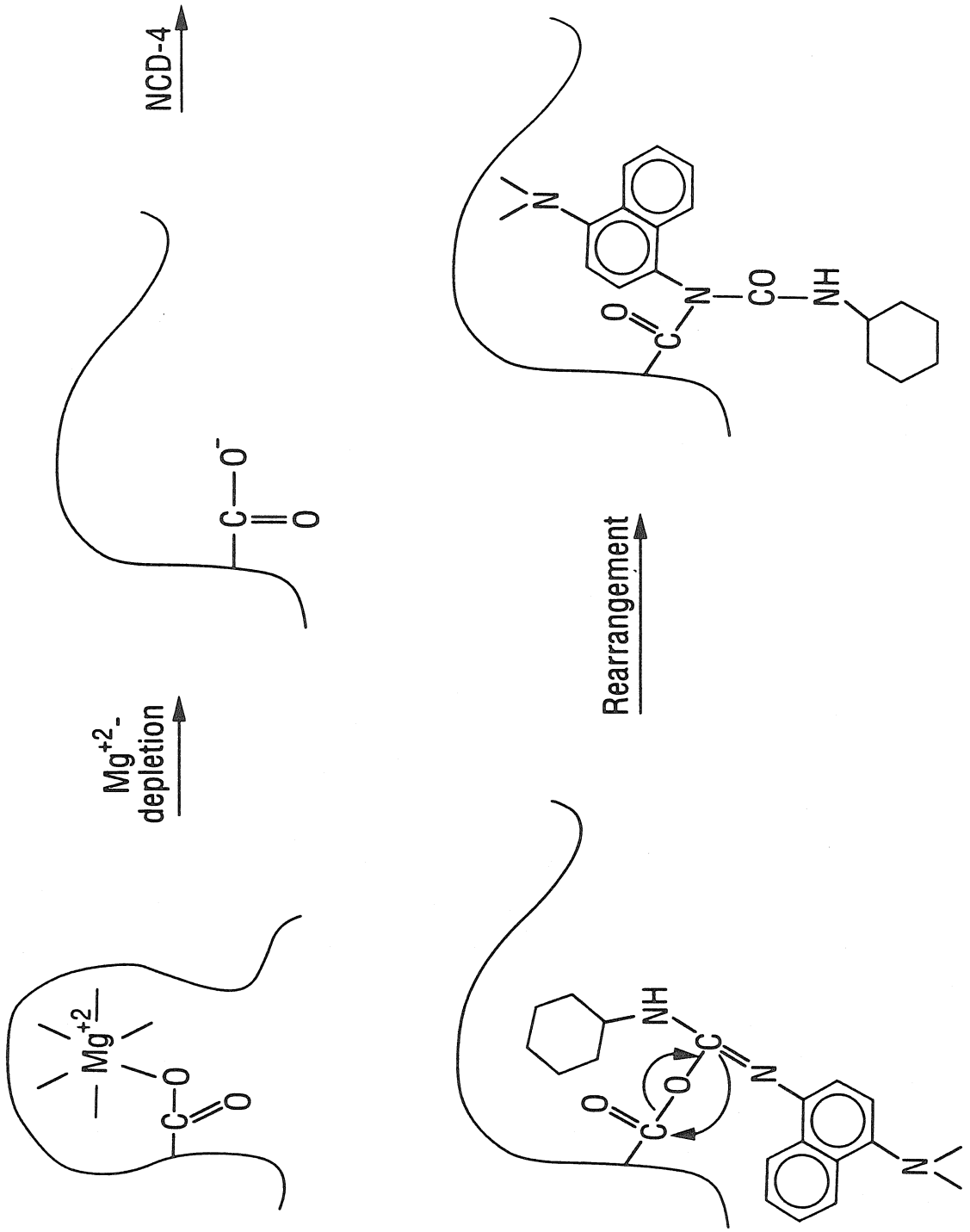
TABLE II

Effect of heat treatment and magnesium-depletion on cytochrome c oxidase activity

Preparation	Activity (%)
Native	100
Heat-treated	75
Heat treated and dialyzed against EDTA (25% heat-treated enzyme + 75% Mg^{+2}-depleted enzyme)	49
Magnesium-depleted	40[a]

[a] Activity of the magnesium-depleted enzyme was obtained from the change in activity of the heat-treated enzyme subsequent to 75% magnesium-depletion.

Figure II.1. NCD-4-labeling of a carboxyl ligand of the magnesium ion in cytochrome *c* oxidase following magnesium depletion.


NCD-4
Mg+2-depletion
Rearrangement
Mg+2
C—O
C—O-
O
NH
CO
N

methionine 123, which consists of several carboxyl containing amino acids. In the present work, NCD-4 was chosen because upon reaction with a carboxyl, it undergoes a rearrangement to N-acylurea, which is fluorescent (λ_{EXmax} = 325 nm; λ_{EMmax} = 440 nm, in 1:1 v/v ethanol-water mixture) (Chadwick *et al.*, 1983) (Fig. 1). This fluorescence provides a convenient handle to follow the outcome of the NCD-4 labeling.

The effect of NCD-4 modification of native, heat-treated and magnesium-depleted CcO is shown in Figure II.2. As shown in lane 1, NCD-4 modifies subunits III and IV in native oxidase. The NCD-4 labeling of subunit III is more extensive than that of subunit IV. Similar differential labeling of subunits III and IV in native oxidase has previously been noted by Casey *et al.* (Casey *et al.*, 1979) with DCCD. The NCD-4 labeling of the heat-treated oxidase (lane 2) reveals no noticeable changes in the labeling pattern or relative intensity. On the other hand, upon magnesium-depletion (lane 3), the NCD-4 labeling of subunit IV becomes significantly enhanced relative to what is observed for the native and heat-treated oxidase. We have obtained no evidence to suggest that the magnesium depletion process (dialysis with EDTA) has increased the assessibility of the carboxyl ligands of subunits I, II, III as well as those of the smaller subunits (V, VI, and VII) toward NCD-4 labeling. The logical conclusion from these data is that one or more carboxyl group(s) in subunit IV has become exposed and available for chemical modification by NCD-4 upon dissociation of the magnesium from the enzyme. It is possible that one or more of these carboxyls are ligands of the magnesium ion, although the possibility of other indirect effects of the magnesium depletion on the NCD-4 labeling of subunit IV could not be excluded.

Sequence analysis—In Figure II.3, we show the sequences and sequence alignments of subunit IV from four different mammalian species (human, bovine, rat, and mouse) and their counterparts in two lower eucaryotes (subunit V of *Neurospora crassa* CcO and subunit Va and Vb of *Saccharomyces cerevisiae* CcO). This sequence comparison reveals four carboxyl amino acids that are conserved among these peptides (outlined). Interestingly, despite the low overall homology of

Figure II.2. NCD-4 labeling of the native, heat-treated, and magnesium-depleted oxidase. The subunit IV of magnesium-depleted enzyme is labeled more strongly than that of native and heat-treated enzyme. On the left, the subunits on the gels are monitored by fluorescence emission; on the right, the subunits are discerned by Coomassie Blue stain. Lane 1: native; lane 2: heat-treated; lane 3: magnesium-depleted.

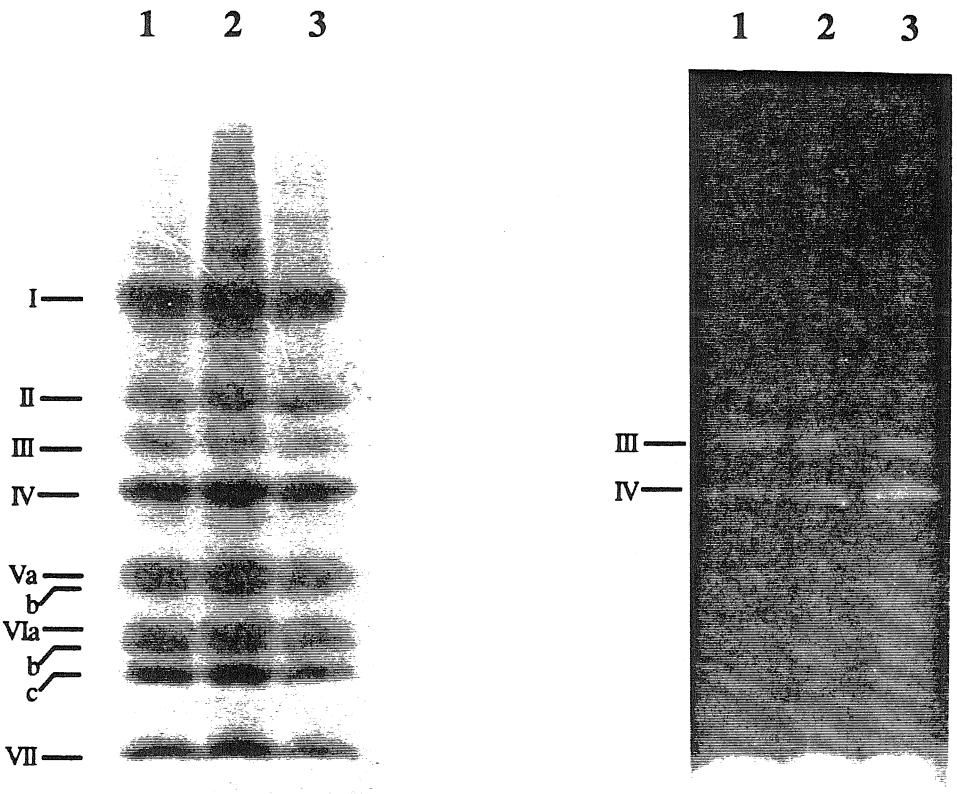
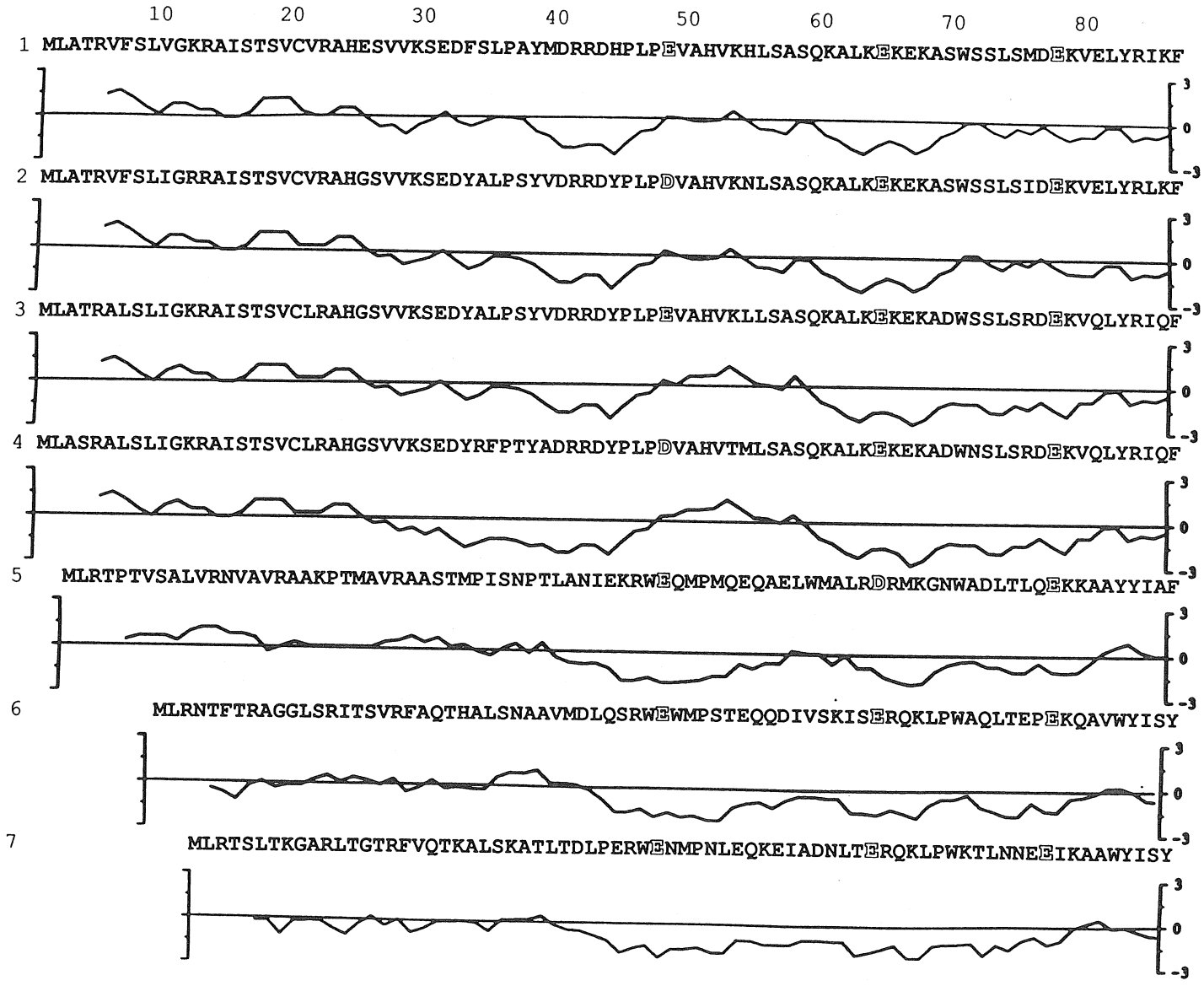

1
2
3
I
II
III
IV
Va
b
VIa
b
c
VII
1
2
3
III
IV

Figure II.3. Sequences, sequence alignments, and hydropathy analysis of CcO subunit IV's from four different mammalian species and their counterparts in two lower eucaryotes. 1. subunit IV of Human CcO; 2. subunit IV of Bovine CcO; 3. subunit IV of Rat CcO; 4. subunit IV of Mouse CcO; 5. subunit V of *Neurospora crassa* CcO; 6. subunit Va of *Saccharomyces cerevisie* CcO; 7. subunit Vb of *Saccharomyces cerevisie* CcO.


10 20 30 40 50 60 70 80
1 MLATRVFSLVGKRAISTSVCVRAHESVVKSEDFSLPAYMDRRDHPLPEVAHVKHLSASQKALKEKEKASWSSLSMDEKVELYRIKF
2 MLATRVFSLIGRRAISTSVCVRAHGSVVKSEDYALPSYVDRRDYPLPDVAHVKNLSASQKALKEKEKASWSSLSIDEKVELYRLKF
3 MLATRALSLIGKRAISTSVCLRAHGSVVKSEDYALPSYVDRRDYPLPEVAHVKLLSASQKALKEKEKADWSSLSRDEKVQLYRIQF
4 MLASRALSLIGKRAISTSVCLRAHGSVVKSEDYRFPTYADRRDYPLPDVAHVTMLSASQKALKEKEKADWNSLSRDEKVQLYRIQF
5 MLRTPTVSALVRNVAVRAAKPTMAVRAASTMPISNPTLANIEKRWEQMPMQEQAELWMALRDRMKGNWADLTLQEKKAAYYIAF
6 MLRNTFTRAGGLSRITSVRFAQTHALSNAAVMDLQSRWEWMPSTEQQDIVSKISERQKLPWAQLTEPEKQAVWYISY
7 MLRTSLTKGARLTGTRFVQTKALSKATLTDLPERWENMPNLEQKEIADNLTERQKLPWKTLNNEEIKAAWYISY
3
0
-3

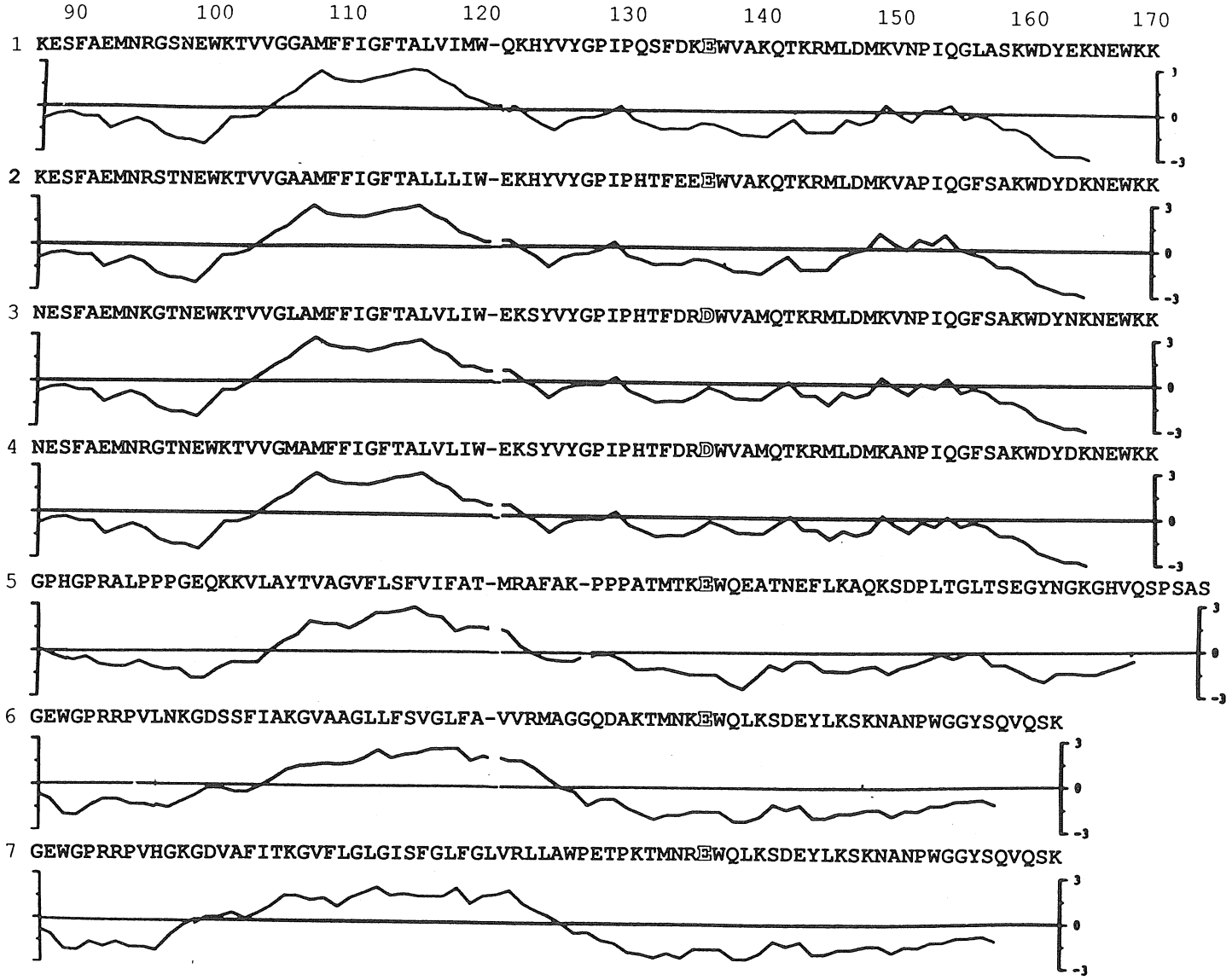
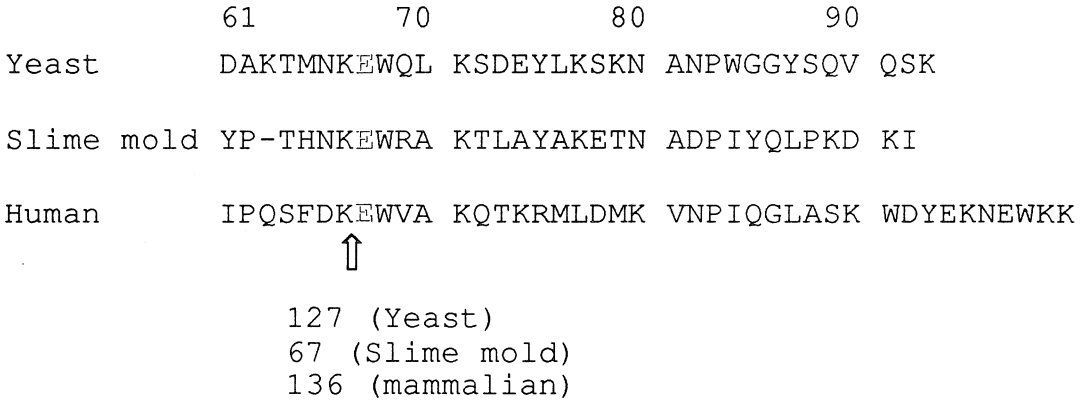

90 100 110 120 130 140 150 160 170
1 KESFAEMNRGSNEWKTVVGGAMFFIGFTALVIMW-QKHYVYGPIPQSFDKEWVAKQTKRMLDMKVNPIQGLASKWDYEKNEWKK
2 KESFAEMNRSTNEWKTVVGAAMFFIGFTALLLIW-EKHYVYGPIPHTFEEEWVAKQTKRMLDMKVAPIQGFSAKWDYDKNEWKK
3 NESFAEMNKGTNEWKTVVGLAMFFIGFTALVLIW-EKSYVYGPIPHTFDRDWVAMQTKRMLDMKVNPIQGFSAKWDYNKNEWKK
4 NESFAEMNRGTNEWKTVVGMAMFFIGFTALVLIW-EKSYVYGPIPHTFDRDWVAMQTKRMLDMKANPIQGFSAKWDYDKNEWKK
5 GPHGPRALPPPGEQKKVLAYTVAGVFLSFVIFAT-MRAFAK-PPPATMTKEWQEATNEFLKAQKSDPLTGLTSEGYNGKGHVQSPSAS
6 GEWGPRRPVLNKGDSSFIAKGVAAGLLFSVGLFA-VVRMAGGQDAKTMNKEWQLKSDEYLKSKNANPWGGYSQVQSK
7 GEWGPRRPVHGKGDVAFITKGVFLGLGISFGLFGLVRLLAWPETPKTMNREWQLKSDEYLKSKNANPWGGYSQVQSK

Figure II.3 also shows the results of a Kyte-Doolittle hydropathy analysis of these peptides. It is evident from this analysis that these peptides exhibit very similar hydropathy properties in the region from amino acid 58 to 140. Within this domain are three carboxyls containing amino acid, namely amino acids 64, 77, and 136 in mammalian CcO. All three carboxyls reside in relatively hydrophilic environments and are flanked by other hydrophilic amino acids. Accordingly, these three conservative carboxyls are good candidates for the ligand(s) of the magnesium ion. In contrast, the remaining conservative carboxyl in these sequences, namely amino acid 48 of mammalian CcO exhibits a broader range of hydropathy environments from species to species. It is, therefore, very unlikely that this carboxyl corresponds to one of the binding ligands of the magnesium ion.

According to the recent study of Rizzuto *et al.* (Rizzuto *et al.*, 1991), subunit VI of cytochrome c oxidase from the slime mold (*Dictyostelium discoideum*) also shows homology with subunit IV of mammalian CcO and subunit Va of yeast CcO. Sequence comparision indicates a significant degree of similarity between the C-terminal part of subunit VI of *Dictyostelium discoideum* CcO (from amino acid 62 to 87) and a segment of subunit Va of yeast CcO (from amino acid 120 to 145) (42.3% identity) as well as a segment of subunit IV of human CcO (from amino acid 129 to 154) (34.6% identity). In particular, a conservative carboxyl amino acid (amino acid 67) has been identified toward the C-terminus of Slime mold, which corresponds to amino acid 136 in the mammalian enzyme. Hydropathy analysis of these segments (Rizzuto *et al.*, 1991) reveals a hydrophobic stretch (about 20 amino acids) flanked by hydrophilic domains. This hydrophobic

Figure II.4. Sequence alignment of C-terminus (From amino acid 60 to 92 of slime mold subunit VI) of subunit Va of Yeast CcO, subunit VI of Slime mold CcO and subunit IV of Human CcO.


61 70 80 90
Yeast DAKTMNKEWQL KSDEYLKSKN ANPWGGYSQV QSK
Slime mold YP-THNKEWRA KTLAYAKETN ADPIYQLPKD KI
Human IPQSFDKEWVA KQTKRMLDMK VNPIQGLASK WDYEKNEWKK
127 (Yeast)
67 (Slime mold)
136 (mammalian)

stretch could be a putative membrane spanning anchor (Zhang *et al.*, 1988). As the conservative carboxyl (Glu 67 in subunit VI of *Dictyostelium discoideum* CcO and Glu/Asp 136 in subunit IV of mammalian CcO) lies in the C-terminus of their respective subunits and these domains exhibit high sequence similarity, this part of protein must be important in the structure and function of the enzyme. Whether this carboxyl is the putative ligand to the magnesium ion remains to be seen. Interestingly, however magnesium is an integral part of the enzyme from *Dictyostelium discoideum* as well.

Our labeling studies with NCD-4 of subunit IV of bovine CcO are consistent with the proposition that the magnesium of mammalian CcO is located in this subunit, as suggested earlier by Yewey *et al.* (Yewey *et al.*, 1987). Sequence comparison suggests Glu 136 as the most likely carboxyl ligand in subunit IV of the mammalian enzyme for the magnesium ion. Since other studies have implicated subunit IV as the binding site for ATP, a picture is gradually emerging on the role of magnesium and subunit IV (mammalian CcO) in the function of this important enzyme.

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Chapter III

The Electron Input from Cytochrome c to Native and Cu_A Modified Cytochrome c Oxidase

ABSTRACT:

The electron input from heme c of cytochrome c to native, type I, type II and Cu_A-depleted CcO under physiological ionic strength has been studied by the laser flash-photolysis technique with 5-deazariboflavin and EDTA as the electron donor. Biexponential kinetics was observed on all four samples. While the rate of the slow phase are unchanged among these four samples (slower than 80 s^{-1}), the rate constant for fast phase showed significant difference. The rate constants of the fast phase for these four samples are 2,580 s^{-1} (native), 2,000 s^{-1} (type I), 990 s^{-1} (type II), 740 s^{-1} (Cu_A-depleted). Increasing Cu_A center perturbation decreases the electron input rate constant to heme a. The decrease of the electron input rate to heme a is not due to a perturbation of the cytochrome c and CcO binding, as the binding affinity between cytochrome c and CcO is not influenced by the modification. The results suggest that electron input from cytochrome c could take place through either Cu_A or heme a, directly. The electron inputted into Cu_A will be subsequently transfer to heme a with a rate faster than the input rate. The first electron inputted from cytochrome c is deposited to heme a before it is transfered to the binuclear center.

INTRODUCTION:

Cytochrome c oxidase (CcO), the terminal enzyme of the respiratory chain in eukaryotes, mediates the transfer of electrons from cytochrome c to dioxygen in mitochondrion. When ferrocytochrome c is oxidized, dioxygen is reduced, protons are pumped vectorically from the mitochondrial matrix to the cytosol. The free energy resulted from this redox chemistry is converted into an electrochemical potential across the inner membrane of the mitochondrion, which ultimately drives the F_0/F_1 ATP synthase to produce ATP.

CcO is a metalloprotein with a number of metallic centers. Two irons (heme a and heme a_3), two coppers (Cu_A and Cu_B) form the redox centers of each CcO monomer. One zinc and one magnesium also associate with each monomer. CcO usually existed as a dimmer with a copper (Cu_X) to stabilize the dimmer structure (Pan *et al.*, 1991). The mammalian enzyme consists of 13 subunits with a molecular weight about 200 KDa (Kadenbach *et al.*, 1983). The three largest subunits are encoded by mitochondrial genes and they are responsible for the electron transfer and proton pumping activities of the enzyme. Heme a, heme a_3 and Cu_B are located in subunit I. Cu_A is located in subunit II, which also provides the binding site for cytochrome c. It is now established that the dioxygen chemistry takes place at a binuclear cluster consisting of heme a_3 and Cu_B. The role of heme a and Cu_A in the overall function of the enzyme has long been debated, but there is a general consensus that heme a and Cu_A mediate the flow of electron from cytochrome c to the dioxygen reduction site. Whether both heme a and Cu_A are capable of accepting electrons directly from cytochrome c, or the electron input into the enzyme occurs at only one of these centers was not clear before. If electron input into enzyme occurs at only one of these centers, then which one of the two low potential centers is the primary electron acceptor is still controversial. Cytochrome c is about 5 Å away from Cu_A compared to a distance of 20-25 Å (Alleyne and Wilson, 1987) from heme c to heme a. Therefore, Cu_A should be a more favorable initial electron acceptor, based on the consideration of the distance for electron transfer.

The intermolecular electron transfer between cytochrome c and CcO has attracted considerable attention in recent years (Hill and Greenwood, 1984; Wilson *et al.*, 1975; Ahmad *et al.*, 1982; Antalis and Palmer, 1982; Hazzard *et al.*, 1991; Hill, 1991). The determination of the initial electron acceptor is of particular interest because of the possible involvement of one of these two centers in proton pumping (Babcock and Callahan, 1983; Gelles *et al.*, 1986). In this report, we investigate the influence of Cu_A site modification on the electron input from cytochrome c to CcO. Our results support that the Cu_A and heme a all involve in accepting the first electron from cytochrome c, although electron transfer to Cu_A center is more facile.

MATERIALS AND METHODS:

Material. Horse heart cytochrome c (type VI), p-(hydroxymercuri) benzoate (pHMB) were obtained from Sigma Chemical Co. (St. Louis, MO). CcO was isolated and purified from bovine heart mitochondria according to the method of Hartzell and Beinert (1974). The enzyme preparation was stored at -78°C before use. Enzyme concentrations were determined from the absorbance change $\Delta A_{red\text{-}ox}$ at 605 nm using an extinction coefficient of 24 mM^{-1} (Van Gelder, 1966).

Transient electron transfer from cytochrome c to CcO. Electron transfer was initiated by the laser flash photolysis technique developed by Hazzard *et al* (Hazzard *et al.*, 1991). When 5-deazariboflavin (5-DRF) is excited by a laser flash, it abstracts an electron from EDTA and becomes a flavin semiquinone. The flavin semiquinone rapidly reduces cytochrome c, which subsequently reduces CcO at a slower rate. The reoxidation of reduced cytochrome c by the oxidase was followed by monitoring the 550 nm absorption peak of the cytochrome c. The electron transfer rate was also followed by monitoring the reduction of cytochrome a of CcO at 604 nm. These experiments were performed on a solution containing 20 μM cytochrome c and 10-50 μM of

oxidase in a buffer of 5 mM Tris containing 1 mM EDTA, 0.1% lauryl maltoside, 50 μM 5-DRF, and 100 mM KCl, pH 7.4. The sample cuvette was sealed and degassed before being subjected to the excimer dye laser flash (BBQ at 395 nm). The optical signals represented the accumulation of 50 flashes.

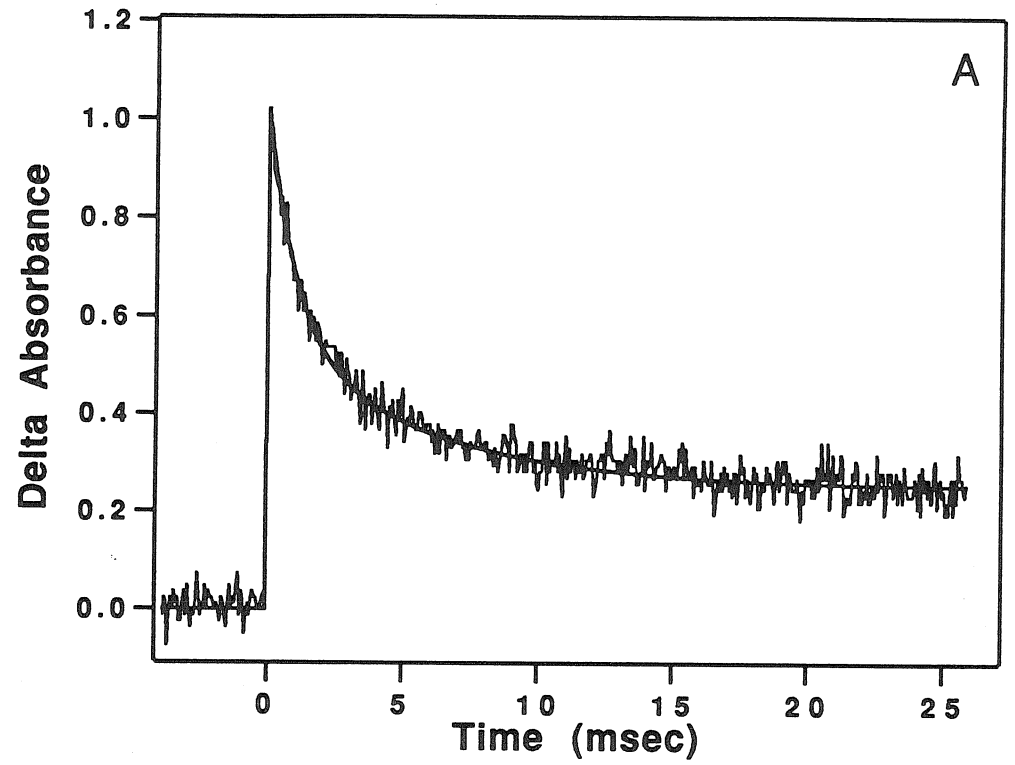
Preparation of type I, type II Cu and Cu_A depleted CcO. Three Cu_A site modification samples are prepared. Type I " blue" copper is prepared by heat treatment of cytochrome c oxidase at 40° C for 1 hr, which results in the lost of one cysteine ligand of Cu_A. Type II copper is prepared according to the method of Nilsson, T. *et al.* (1988) with p-(hydroxymercuri) benzoate (pHMB) modification of the thiols and resulting in loss of all cysteine ligand of Cu_A. Cu_A depletion sample is prepared by the method of Li, P. M. *et al.* (1987), in which EDTA was used to removed the Cu_A from pHMB modification sample.

RESULTS:

Figure 1 show the kinetic data observed for the intracomplex electron transfer between bovine cytochrome c and fully oxidized native bovine CcO at 1:1 molar ratio and 110 mM ionic strength. The reduction of ferricytochrome c by 5-DRF semiquinone and its subsequent reoxidation by CcO were monitored at 550 nm (Figure III.1A). The kinetic trace is biphasic and fits well to a sum of two exponential. A rate constant of 1250±63 s^{-1} is obtained for the fast phase with an amplitude corresponding to 75 % of the total signal change. The reduction of cytochrome a was followed at 604 nm (Figure III.1B). This reduction is also biphasic with a rate constant of 1300± 45 s^{-1} for the fast phase. Thus, there is excellent correspondence between the reoxidation of the ferrocytochrome c and the reduction of cytochrome a in the fast phase.

Under otherwise identical conditions, the type I, type II and Cu_A-depleted CcO also displays biphasic kinetics for the reoxidation of ferrocytochrome c and reduction of cytochrome a (Figure

Figure III.1. Intracomplex electron transfer between cytochrome c reduced by photogenerated flavin semiquinone and native CcO. 20 μM native bovine CcO and 20 μM bovine cytochrome c were added in 5 mM Tris buffer at pH 7.4 containing 1 mM EDTA, 0.1 % lauryl maltoside, 100 μM 5-DRF, and 100 mM KCl. The sample cuvette was degassed and subjected to a N_2 dye laser (BBQ at 390 nm) flash. The signals are the sum of four flashes and normalized to one. (A) Reduction of cytochrome c by flavin semiquinone and reoxidation of ferrocytochrome c was followed at 550 nm. (B) Reduction of cytochrome a by the ferrocytochrome c was observed at 604 nm. Both transients were fitted to a sum of two exponentials (solid curves).


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

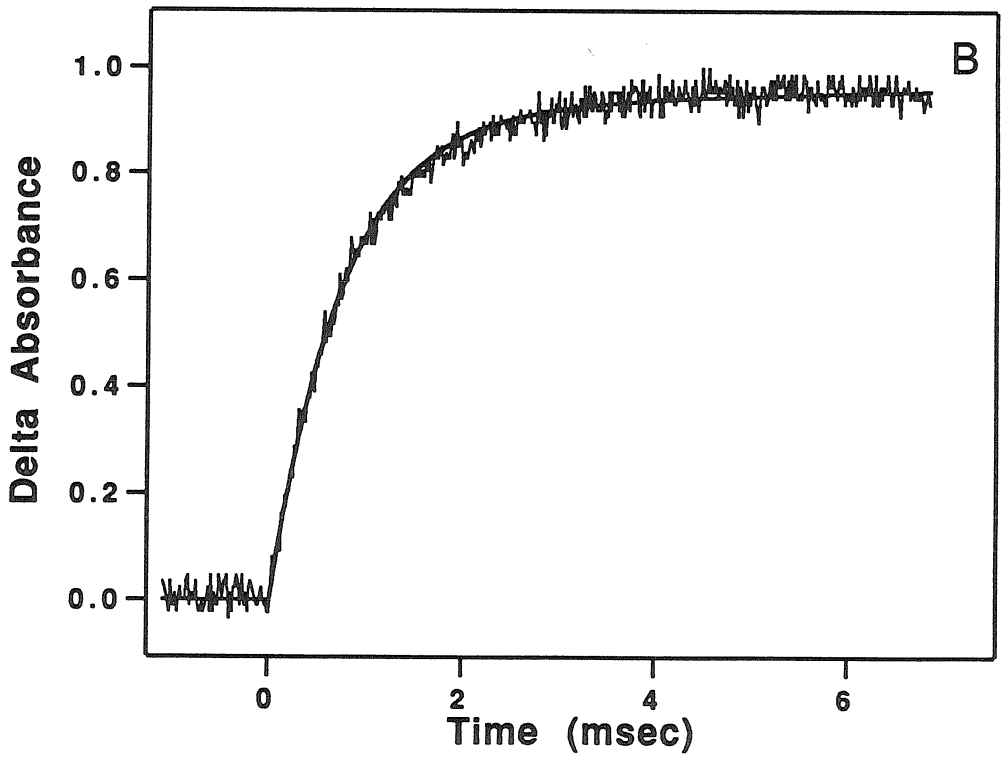
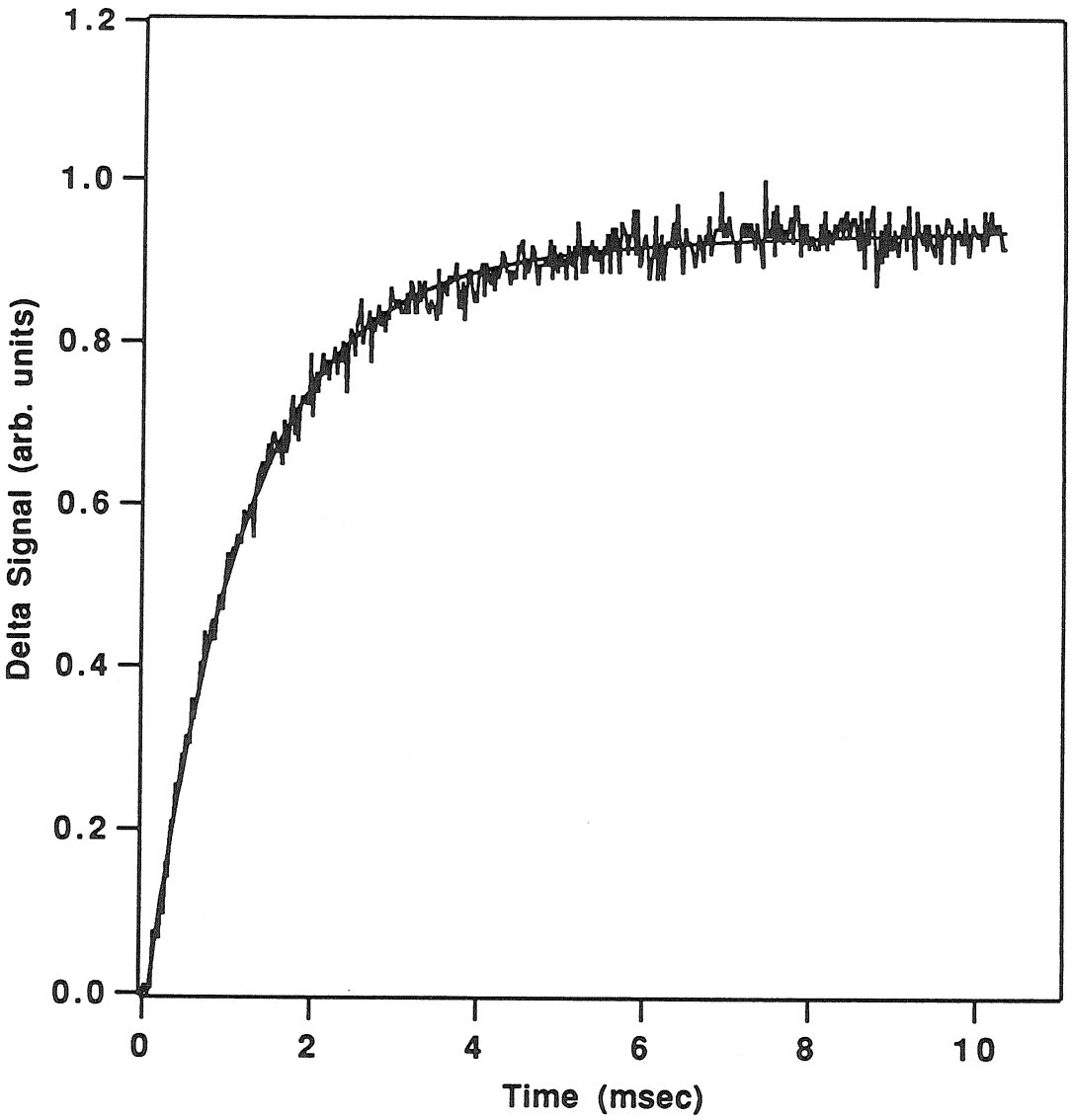

B
Delta Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
Time (msec)

Figure III.2. Intracomplex electron transfer between cytochrome c reduced by photogenerated flavin semiquinone and type I copper CcO. The experimental conditions are identical to those in Figure 1. Solid curves depict double-exponential fits to the data.


1.2
1.0
0.8
0.6
0.4
0.2
0.0
Delta Signal (arb. units)
0
2
4
6
8
10
Time (msec)

2, 3 and 4). Whereas the rate constants for the slow phase are the same for both the native, type I, type II and Cu_A-depleted enzymes (slower than 80 s^{-1}), the rate constant for the fast phase for the Cu_A-depleted protein is approximately 25% that of the native enzyme. The fitting gives 300 ± 20 s^{-1} (at 604 nm) and 320 ± 18 s^{-1} (at 550nm). For type I and type II enzyme the rate constant for the fast phase is approximately 75% and 33% that of the native enzyme respectively. The fittings for type I and type II give 890 s^{-1} and 400 s^{-1} respectively. The rate constant for the fast kinetic phase (k_{obsd}) depends on the concentration of CcO. This dependence is hyperbolic for all the native, type I, type II and Cu_A-depleted sample (Figure III.5 and 6), suggesting that cytochrome c oxidase reduction proceeds via a mechanism in which a 1:1 transient complex is formed between ferrocytochrome c and cytochrome c oxidase:

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

$$\text{Cyt } c^{2+}\text{: } (CcO)_{ox} \xrightarrow[k_{et}]{} \text{Cyt } c^{3+} + (CcO)_{red} \qquad (2)$$

where k_1 is the reaction of the reduced cytochrome c with oxidized cytochrome c oxidase to form a transient complex, k_2 is the dissociate rate of this complex, k_{et} is the electron transfer rate from reduced cytochrome c to CcO within the transient complex. According to this mechanism, we have the expression of the observed rate constant

$$K_{obsd} = k_{et} K_A [\ CcO\ ]_{ox} / (\ K_A [\ CcO\ ]_{ox} + 1\ ) \qquad (3)$$

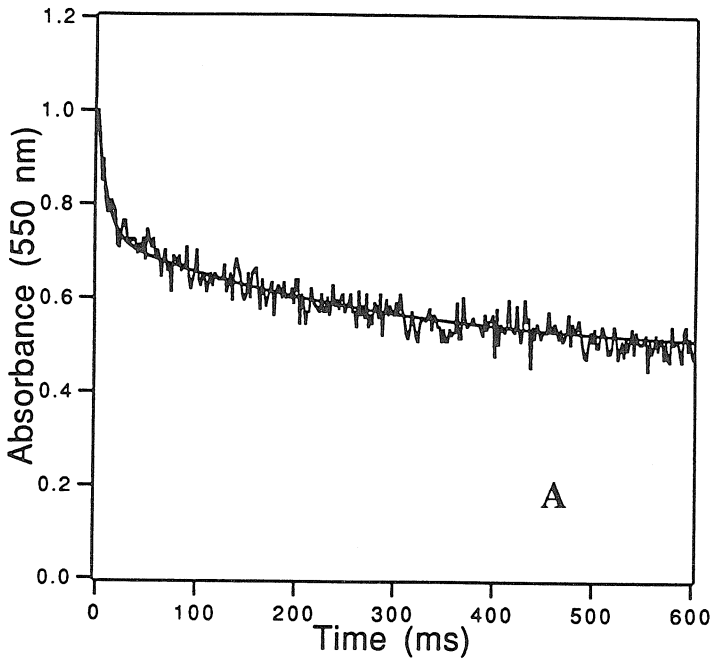
where K_A ($=k_1/k_2$) is the association constant for the formation of the ferrocytochrome c:CcO complex. When concentration of CcO is sufficiently high, the observed rate constant expression above could be simplified to

$$k_{obsd} = k_{et} \qquad (4)$$

k_{obsd} equal to k_{et}, when the concentration of CcO is high enough to saturate the binding of cytochrome c.

The best kinetic data fits for native and Cu_A-depleted samples using the equation 3, shown in figure III.5, give the K_A value for native is $5.4\times10^4 M^{-1}$ and for Cu_A-depleted is $5.0\times10^4 M^{-1}$. The

Figure III.3. Intracomplex electron transfer between cytochrome c reduced by photogenerated flavin semiquinone and type II copper CcO. The experimental conditions are identical to those in Figure 1. Solid curves depict double-exponential fits to the data.


1.2
1.0
0.8
0.6
0.4
0.2
0.0
Absorbance (550 nm)
A
0
100
200
300
400
500
600
Time (ms)

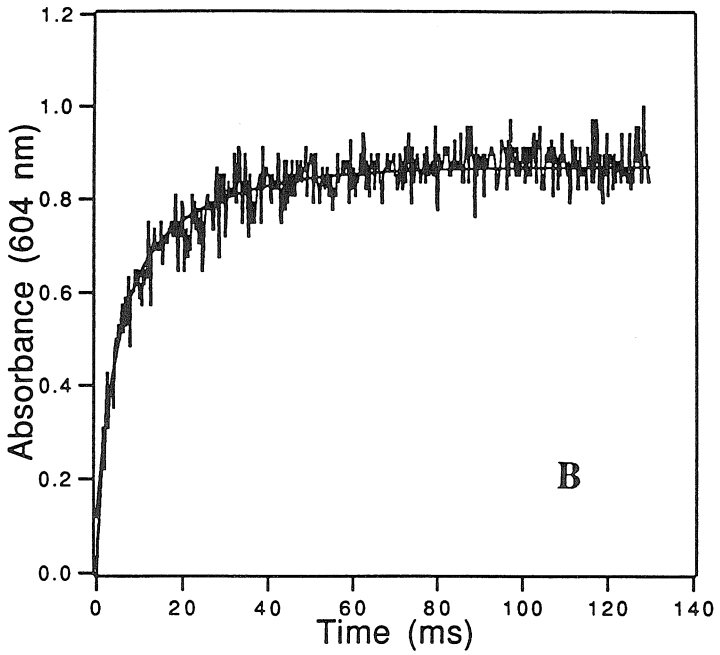
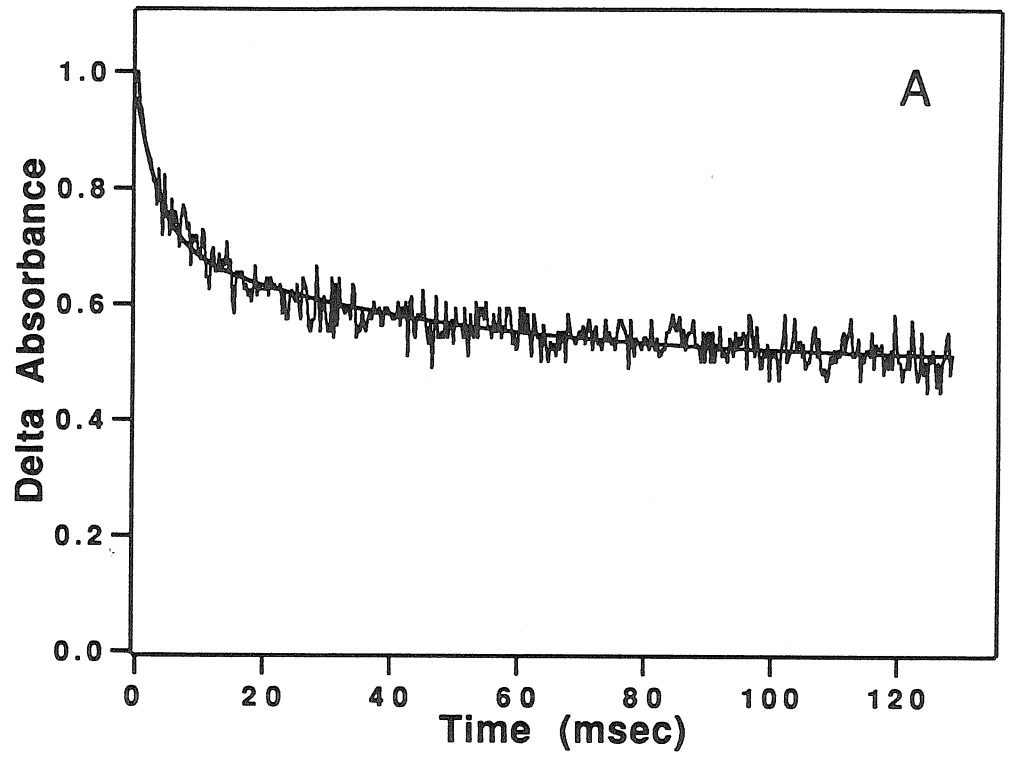

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Absorbance (604 nm)
B
0
20
40
60
80
100
120
140
Time (ms)

Figure III.4. Intracomplex electron transfer between cytochrome c reduced by photogenerated flavin semiquinone and Cu_A-depleted CcO. The experimental conditions are identical to those in Figure 1. Solid curves depict double-exponential fits to the data.


A
Delta Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
Time (msec)

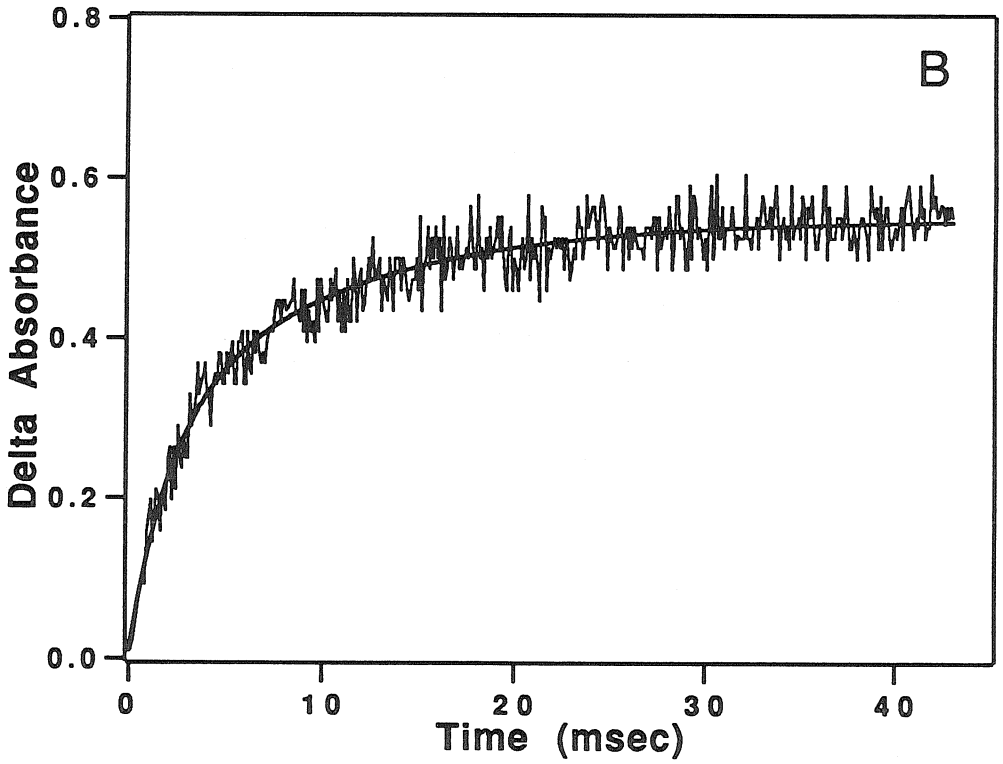
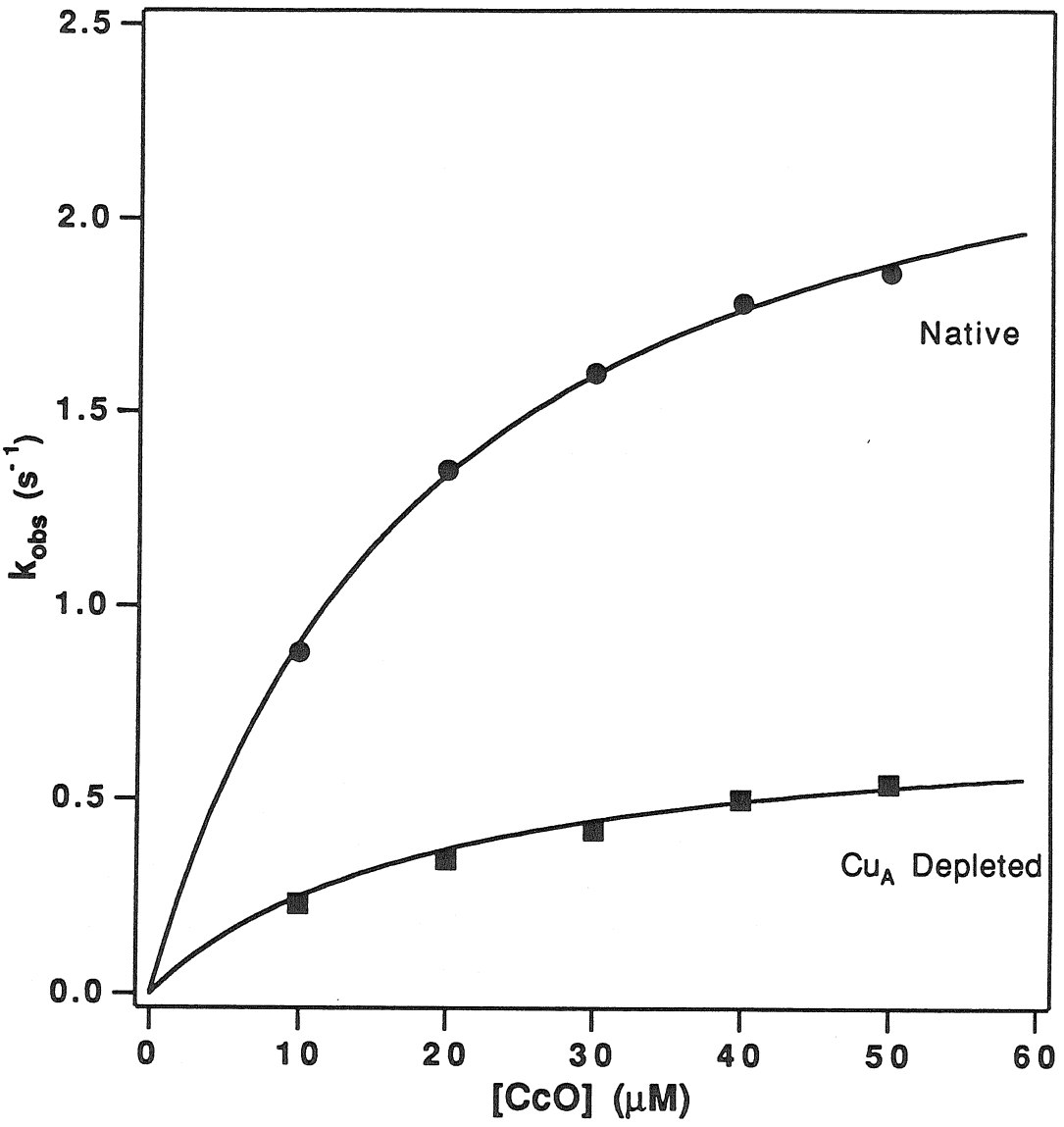

B
Delta Absorbance
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
Time (msec)

Figure III.5. Kinetics of electron transfer between ferrocytochrome c and native and Cu_A-depleted CcO at various CcO concentrations and ionic strength of 110 mM. The reaction conditions are as shown in Figure 1 and the concentration of cytochrome c is unchanged as the concentration of CcO is varied. 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 CcO. The solid curves represent the best fits of the data to equation (3).


2.5
2.0
1.5
1.0
0.5
0.0
k_{obs} (s^{-1})
Native
Cu_A Depleted
0
10
20
30
40
50
60
[CcO] (μM)

best kinetic data fits for type I and type II copper CcO shown in figure 6 give the K_A value for type I is $4.2\times10^4M^{-1}$ and for type II is $4.0\times10^4M^{-1}$. All these K_A values are presented in Table 1. The K_A values of these three Cu_A modification samples are close to that of native enzyme. This indicates that these Cu_A site modifications do not greatly change the conformation of CcO, and therefore do not disrupt the formation of complex between cytochrome c and CcO. The kinetic data fits using equation 3 for native and Cu_A-depleted samples shown in figure III.5 and for type I and type II in figure III.6 also give the electron transfer rate for each sample which is shown in Table 1. The rates of intracomplex electron transfer are significantly different for native and these Cu_A modification oxidases. The intracomplex electron transfer rate is the fastest for native enzyme and decrease from type I, type II to Cu_A-depleted CcO with increasing perturbation of Cu_A site. For the Cu_A depletion CcO, the electron input from cytochrome c to CcO is still observed but at a greatly slower rate.

The overall electron transfer stoichiometries in these experiments can be deduced from the observed signals, which were normalized and shown in figure III.1, 2, 3 and 4. To calculate the reaction stoichiometries, the observed signal changes at 604 and 550 nm were converted to delta absorbance by using the appropriate scaling factor, after correcting for the percent attenuation changes in ferrocytochrome c at each wavelength (26% and 50 %, respectively). The concentration changes in ferrocytochrome c and reduced cytochrome a were determined by using $\Delta\varepsilon$=20 $mM^{-1}cm^{-1}$ and $\Delta\varepsilon$=22 $mM^{-1}cm^{-1}$, respectively. A 1:1 molar ratio of ferrocytochrome c reoxidized to cytochrome a reduced was obtained for the native (1.13 μM/1.09 μM), Cu_A-depleted (0.48 μM/0.50 μM), type I copper (0.97 μM/0.95 μM) and type II copper (0.31μM/0.33μM) sample (Table 2). The single electron input from cytochrome c to CcO is the expected result under our experimental conditions, since the extent of the reaction is limited by the number of reducing equivalents generated by the laser excitation of 5-DRF. In these experiments, only about 5 % of the cytochrome c is reduced by the laser flash, and these reducing equivalents are rapidly consumed by CcO in single electron transfer from the ferrocytochrome c to the CcO because of the high specific

Table 1. Kinetic parameters for intracomplex ET between cytochrome c and cytochrome c oxidase

Sample	K_a [x 10^{-4} (M^{-1})]	k_{et} (s^{-1})
native	5.4	2580
type I Cu_A	4.2	2000
type II Cu_A	4.0	990
Cu_A-depleted	5.0	740

Figure III.6. Kinetics of electron transfer between ferrocytochrome c and type I and type II copper CcO at various CcO concentrations and ionic strength of 110 mM. The reaction conditions are as shown in figure 1 and the concentration of cytochrome c is unchanged as the concentration of CcO is varied. 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 CcO. The solid curves represent the best fits of the data to equation (3).

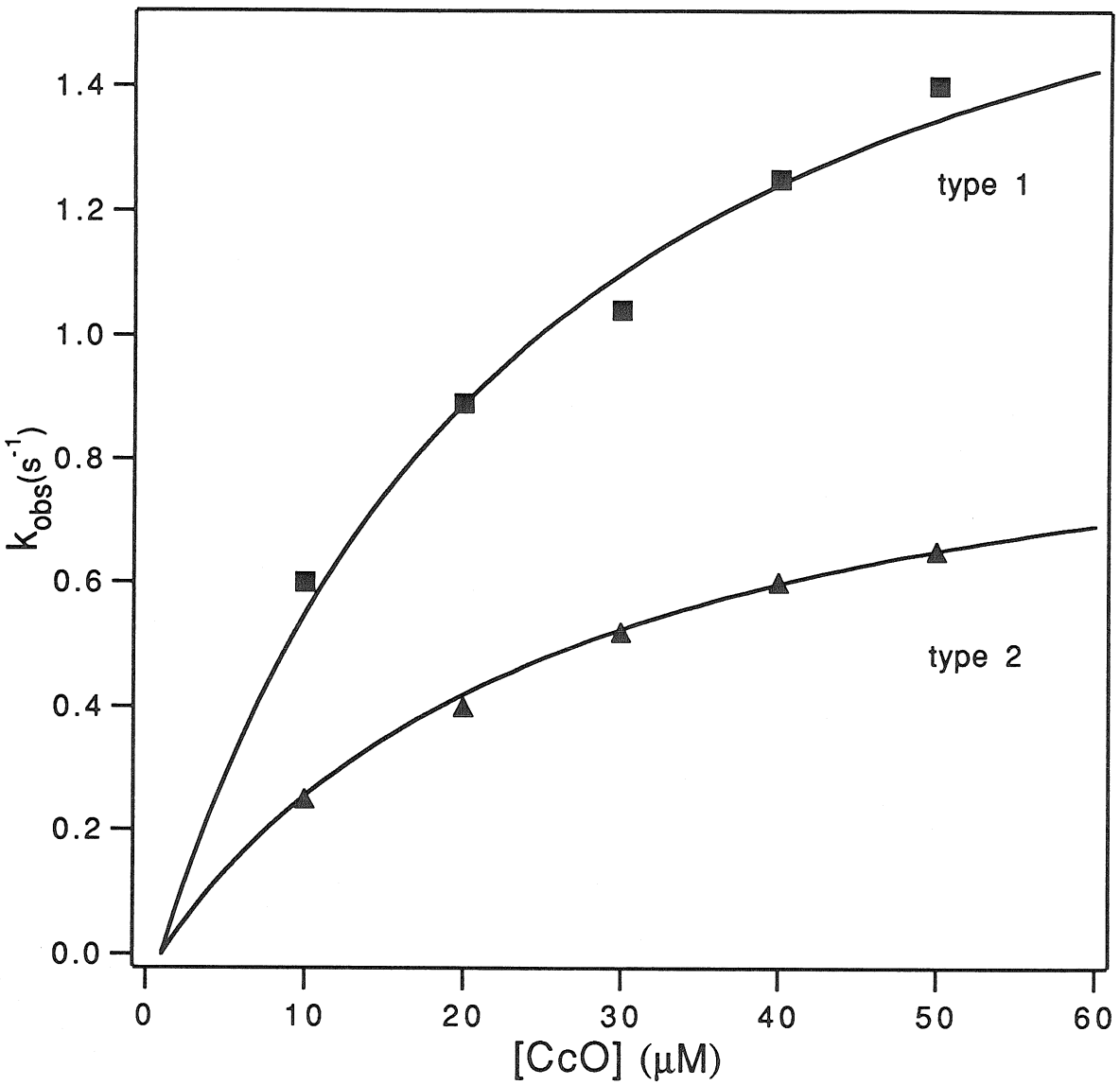

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
$k_{obs}(s^{-1})$
type 1
type 2
0
10
20
30
40
50
60
[CcO] (μM)

Table 2. Changes in Heme a and Heme c Concentration*

Sample	[CcO]/[c]	Δ[a] (μM)	Δ[c] (μM)	Δ[c]/Δ[a]
native	0.5/1	1.09	1.13	1.0
type I CuA	0.5/1	0.95	0.97	1.0
	1/1	1.0	0.9	0.9
type II CuA	0.5/1	0.33	0.31	0.94
CuA-depleted	0.5/1	0.5	0.48	1.14

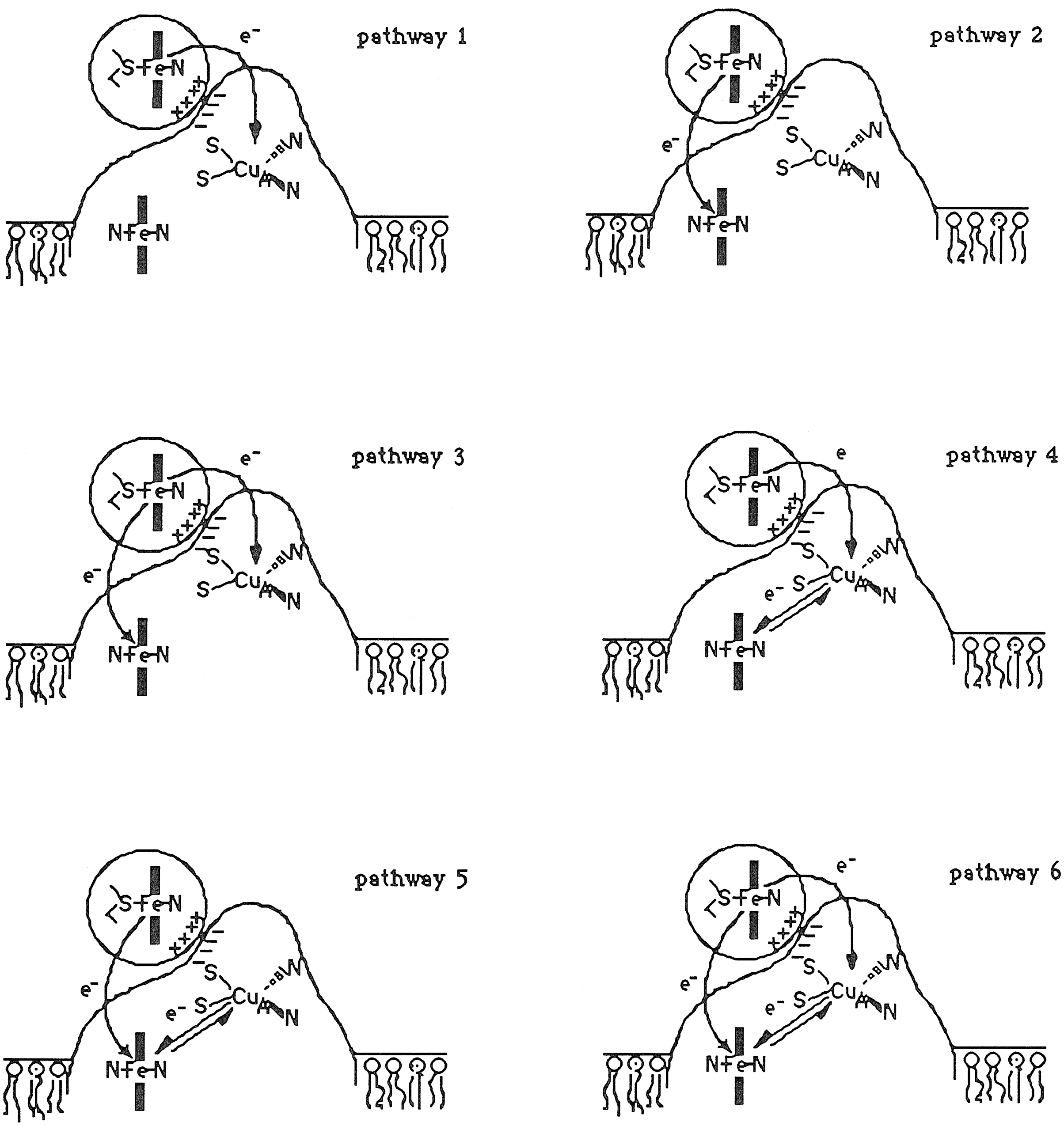
* Determination of the change in [a] and [c] were made based on the ΔS_{604} and ΔS_{550}, respectively, adjusting for the percent attenuation at each wavelength (26 % and 50 % attenuation, respectively), then dividing by a scaling factor to convert to ΔAbsorbance. Δ[a] and Δ[c] are calculated from the ΔA values using $\Delta\varepsilon_{604}$ = 20 $mM^{-1}cm^{-1}$and $\Delta\varepsilon_{550}$ = 22 $mM^{-1}cm^{-1}$.

activity of our enzyme. The 1:1 molar ratio of cytochrome c reoxidation to heme a reduction suggests the electron input to these two low potential centers is deposit to heme a center before it is transferred to high potential center (heme a_3 and Cu_B)

DISCUSSION:

The electron input from cytochrome c to CcO could take place by six possible pathways, based on the possible combinations of three metal centers (heme c, heme a and Cu_A) (Figure III.7). Among these six possible pathways, three of them represent the electron input from heme c to either Cu_A or heme a or both centers without the fast electron exchange between Cu_A and heme a. The remaining three pathways correspond to the first three pathways, but with fast electron exchange between the low potential centers. For the first pathway, electron inputs from cytochrome c to Cu_A site and resides in this center, as there are not fast enough electron equilibrium between Cu_A and heme a. By this pathway, the first electron input to CcO will only reduce Cu_A without affecting the redox state of heme a. For the second pathway, electron inputted from cytochrome c to heme a site and resides in heme a, due to the lack of fast electron equilibrium between Cu_A and heme a centers. By this pathway, the first electron input would reduce heme a and without affecting redox state of Cu_A. Any structural perturbation of Cu_A site should not influence the first electron input by this pathway. For the third pathway, the first electron transfers directly to heme a or Cu_A site parallel, controlled by kinetic factors, and resides in these centers without further electron equilibrium between these two centers. By this pathway, the electron transfer rate from cytochrome c to heme a center will not be affected by the Cu_A center perturbation either. For the fourth pathway, the first electron transfers to Cu_A center first, then a fast electron equilibrium between heme a and Cu_A brings the electron to heme a center. By this pathway, the first electron input rate observed by oxidation of heme a should be totally determined by electron input rate to Cu_A. No electron input should be observed from heme c to heme a of Cu_A-depleted CcO with this pathway. For the fifth pathway, the first electron transfers directly to heme a, then a fast electron equilibrium between

Figure III.7. Six possible electron transfer pathways for initial electron transfer from cytochrome c to low potential centers (heme a and Cu_A) of cytochrome c oxidase.


pathway 1
pathway 2
pathway 3
pathway 4
pathway 5
pathway 6
e⁻
SFeN
NFeN
Cu
S
N

heme a and Cu_A will distribute some electron to Cu_A center according to their relative redox potentials. By this pathway, Cu_A site perturbation should not influence the rate of electron input to heme a and only influence the electron distribution in Cu_A site, which depends on the change of redox potential upon Cu_A site perturbation. For the sixth pathway, the first electron inputs to heme a or Cu_A directly, then a fast electron equilibrium will redistribute the electron between these two centers. By this pathway, the rate of heme a reduction could be partially controlled by the electron transfer rate from cytochrome c to Cu_A center, depending on the relative rate of electron input into these two centers.

Present study could reveal which one of these pathway is adopted for electron input from cytochrome c to CcO. If the first electron input to CcO took place by the first pathway, no change of the redox state of heme a would be expected. This is not consistent with our observation, which showed the reduction of heme a by the first electron. If the second or third was the pathway for electron input, the Cu_A center perturbation should not influence the electron input rate to heme a. Therefore, these two pathways are also inconsistent with our observation on native and Cu_A center perturbed samples, that the electron input was greatly influenced by the Cu_A site perturbation. It is obvious that all three pathways, which lack of fast electron equilibrium between Cu_A and heme a, have been proved to be invalid. Existence of fast electron equilibrium between these two low potential centers is essential for this electron input. Our conclusion confirms the early results of Morgan *et al.* (1989) and Kobayashi *et al.* (1989) that the electron transfers from Cu_A to heme a ($2x10^4$ s^{-1}) is much faster than the rate of electron input rate (2,500 s^{-1}) observed.

The remaining three pathways have a fast electron equilibrium between two low potential centers. If the electron inputted from cytochrome c to CcO by the fourth pathway, the electron input rate to heme a would be limited by the rate of electron input to Cu_A center. When Cu_A is depleted, electron input to Cu_A should be terminated. Therefore, the electron input to heme a became impossible. It does not agree with our observation on Cu_A depleted samples, in which the reduction of heme a

still took place, albeit with slow rate. If the electron input to cytochrome c oxidase processed by fifth pathway, then Cu_A site perturbation should not influence the electron input rate to heme a site. This pathway, thus, conflicts with our experimental observation on the Cu_A center perturbation samples. Only the sixth pathway completely agrees with our experimental results. In this pathway, the electron could transfer directly to Cu_A or heme a. Therefore, when Cu_A is depleted, electron could still input to heme a site. The existence of the fast electron equilibrium between heme a and Cu_A facilitates the electron transfer to heme a by allowing the electron input to Cu_A, which is faster due to the shorter distance between heme c and Cu_A, then followed by the electron transfer to heme a. When Cu_A site was perturbed and the electron input to Cu_A site was slowed down, the rate of heme a reduction observed is also slowed down by this pathway. This pathway is, thus, consistent with our experimental observation.

Since there are only one electron input to these two low potential centers (heme a and Cu_A). The 1:1 molar ratio of cytochrome c reoxidized to cytochrome a reduced for native and type 1 copper sample suggests that the first electron input from cytochrome c directly to Cu_A or heme a, followed by redistribution of the electron from Cu_A to heme a, as the redox potential of heme a is higher tan that of Cu_A. The kinetics of first electron input could be written as:

$$\text{Cyt } c^{2+} : Cu_A^{2+} \xrightarrow{k_1} \text{Cyt } c^{3+} : Cu_A^{+} \qquad (5)$$

$$Cu_A^{+} : \text{Cyt } a^{3+} \underset{}{\overset{k_2}{\longleftrightarrow}} Cu_A^{2+} : \text{Cyt } a^{2+} \qquad (6)$$

$$\text{Cyt } c^{2+} : \text{Cyt } a^{3+} \xrightarrow{k_3} \text{Cyt } c^{3+} : \text{Cyt } a^{2+} \qquad (7)$$

expression (5), (6) represent the electron input to heme a via Cu_A center. Expression (7) represents the electron input to heme a directly from cytochrome c. In expression (5) and (6) k_1 is the rate determined step ($k_2 > K_{-2} >> k_1$), the rate of electron input to heme a via Cu_A center could be written as

$v_1 = k_1$ [cyt c: CcO]. (8)

The rate of electron input from cytochrome c directly to heme a could be written as

$v_2 = k_3$ [cyt c: CcO]. (9)

Since the two processes are parallel, the rate of electron input to heme a we observed on native and type I, type II copper CcO is the sum of these two process

$v = v_1 + v_2 = (k_1 + k_3)$ [cyt c: CcO]. (10)

From expression (10), if we know the value of k_3 (rate constant of electron input from cytochrome c directly to heme a), we could calculate the rate of electron transfer from cytochrome c to Cu_A since $k_1 + k_3$ equal to k_{et}. In order to measure the contribution of these two centers (heme a and Cu_A) in the accepting of the first electron from cytochrome c, we assume that the electron input rate to heme a of Cu_A depleted sample corresponds to the rate of expression (7). From this assumption, we have a k_3 value of 740 s^{-1}. From expression (10), we could calculate the rate of electron input to Cu_A center. The resulting k_1 value is 1840 s^{-1}. Comparing the contribution of accepting of electron from cytochrome c by these two centers, we could conclude that the Cu_A is the more important initial electron acceptor in these two low potential centers. But cytochrome a provides the ultimate disposition of the electron prior to subsequent electron transfer to dioxygen reduction center.

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Chapter IV

8-azido-ATP Modification of Cytochrome c: Retardation of Its Electron Transfer Activity to Cytochrome c Oxidase

ABSTRACT:

Horse heart cytochrome c has been modified by 8-azido-ATP and the electron transfer activity of the modified cytochrome c's to bovine heart cytochrome c oxidase (CcO) under physiological ionic strengths has been studied by the laser flash-photolysis technique with 5-deazariboflavin and EDTA as the electron donor. The intermolecular electron transfer between the redox protein partners was shown to be extremely slow. The 8-azido-ATP modified system exhibited less than 5% of the intracomplex electron transfer rate observed between native cytochrome c and CcO under otherwise identical conditions. The binding affinity of the modified cytochrome c was greatly reduced (3 orders of magnitude) at low ionic strengths, however, it was only slightly reduced (by a factor of 2) relative to the native protein at physiological ionic strengths. Thus, the binding affinity of the ATP-cytochrome c adducts is relatively insensitive toward the ionic strength compared to the native enzyme, suggesting that a different docking conformation is assumed by the ATP-cytochrome c adducts in their interaction with the oxidase. Since the redox potential of the modified cytochrome c is close to the value of its native form, we conclude that there has been a change in the docking of the cytochrome c to CcO and the electronic coupling between heme c and Cu_A upon 8-azido-ATP modification.

INTRODUCTION:

In recent years, there has been a growing interest in the mechanisms by which ATP regulates cellular respiration, particularly in the terminal step of electron transport from ferrocytochrome c to molecular oxygen and the coupled redox-linked proton translocation mediated by cytochrome c oxidase (CcO). The effect of ATP on cytochrome c-CcO kinetics was first reported by Margoliash and coworkers (Ferguson-Miller *et al.*, 1976). Kadenbach, Bisson, Montecucco and their coworkers (Huther and Kadenbach, 1986, 1987, 1988; Montecucco *et al.*, 1986; Bisson *et al.*, 1987) have proposed that the control is allosteric, with ATP-binding to the oxidase modulating the details of the enzyme turnover, including the intramolecular rates and the efficiency of biological energy transduction. On the other hand, Wallace and coworkers (Corthesy and Wallace 1986, 1988, Craig and Wallace, 1991, 1993) have suggested that ATP binds to cytochrome c directly, thereby inhibiting the binding of ferrocytochrome c to CcO and/or retarding the intracomplex electron transfer between the redox protein partners. With this hypothesis in mind, Corthesy and Wallace (1986, 1988) have studied the binding of ATP to cytochrome c. They proposed that ATP binds to cytochrome c near the invariant arginine 91. The affinity of cytochrome c for ATP was shown to be moderate; the pK is on the order of 3-4 mM (Craig and Wallace, 1991), close to the ATP concentration in the cytosol under physiological conditions. In apparent support of this proposal, Craig and Wallace (1993) recently succeeded in cross linking the photoaffinity agent 8-azido adenosine 5'-triphosphate or adenosine 5'-triphosphate-2', 3'-dialdehyde to cytochrome c, and demonstrated quite convincingly that these ATP-adducts of cytochrome c showed a much lower ability to restore dioxygen consumption in cytochrome c-depleted mitochondria relative to native cytochrome c. The implication is that when the ATP-adduct is substituted for native cytochrome c in the mitochondrion, there is a significant decrease in the electron flow through the mitochondrial electron transport chain. However, it should be noted that aside from this observation, which may or may not have any bearing on the effects of noncovalent ATP binding, there has been no direct evidence that free ATP, bound to cytochrome c, influences the docking of

cytochrome c to CcO or cytochrome bc_1, not to mention any deleterious effects on the electron transfer activities between these redox protein partners.

Craig and Wallace (1993) attributed the decrease in the electron flow through the mitochondrial electron transport chain to the effect of ATP-modification on the intrinsic electron transfer activity of cytochrome c with its physiological redox partners and/or the affinity of the modified cytochrome c for the inner mitochondrial membrane. Indeed, the decreased electron transfer activity could arise from a change in the docking of the cytochrome c with the cytochrome bc_1 or the CcO, resulting in different electronic coupling between the donor and acceptor in the complex of the redox partners upon ATP-modification. In an attempt to address this issue further, we have examined the effect of ATP-modification of cytochrome c on the kinetics of the electron transfer between ferrocytochrome c and CcO. In this report, we describe the study of the time course of the electron input from 8-azido-ATP modified cytochrome c to CcO using the laser flash-photolysis transient absorption technique. The intermolecular electron transfer between the redox protein partners was shown to be extremely slow. An attempt has been made to pinpoint the origin of the retardation of the electron transfer and its relevance to non-covalent ATP binding to cytochrome c.

MATERIALS AND METHODS:

Material. Horse heart cytochrome c (type VI), CM Cellulose ionic exchange gel, ATP (grade II) and 8-Azido-ATP were obtained from Sigma Chemical Co. (St. Louis, MO). CcO was isolated and purified from bovine heart mitochondria according to the method of Hartzell and Beinert (Hartzell and Beinert, 1974). The enzyme preparation was stored at -78° C before use. Enzyme concentrations were determined from the absorbance change $DA_{red\text{-}ox}$ at 605 nm using an extinction coefficient of 24 mM^{-1} (Van Gelder, 1966).

Modification of cytochrome c with 8-azido-ATP (-ADP). Cytochrome c was modified by 8-azido-ATP according to the method of Craig *et al.* (Craig and Wallace, 1993). The sample was then loaded onto a CM-cellulose column and eluted with a linear gradient of 5-50 mM, of phosphate buffer, pH7.4. Two major peaks (labeled fraction 1 and 2) were obtained, and identified by the optical spectral change in UV region as the ATP-modified product. The subfractions within each peak were pooled and concentrated, and the cytochrome c-ATP adducts were stored at -78°C until use. The modification of cytochrome c with 8-azido-ADP was performed similar to the method of modification with 8-azido-ATP, except that 8-azido-ATP was replaced by 8-azido-ADP. The modified cytochrome c was pooled, concentrated and stored at -78°C until use. The molecular weight of native and 8-azido ATP modified cytochrome c were determined by MALDI Time-of-flight mass spectroscopy on a Voyeger instrument at Protein/Peptide Micro Analytical Facility of California Institute of Technology.

Determination of the affinity of ATP binding to native and 8-azido-ATP (-ADP) modified cytochrome c and the binding constant of native and 8-azido-ATP (-ADP) modified cytochrome c with CcO using rapid filtration method. The affinity of ATP binding to native and ATP(ADP)-cytochrome c adduct was determined by centrifuging a preequilibrated 2 ml solution of 30 μM of ATP with 10 μM of cytochrome c or ATP (ADP)-cytochrome c adduct at 5 mM Tris buffer pH 7.4 in a centricon-10 microconcentrator, which retains the cytochrome c but allows free ATP to pass the membrane, for 20 min at 3°C. After the centrifugation, the concentration of ATP in the filtrate and the original solution together with that of cytochrome c in the original solution were determined by UV-Visible spectra.

To determine the binding constants of the different cytochrome c's to CcO, a preequilibrated solution of 10 μM of cytochrome c or the ATP(ADP)-cytochrome c adduct with 10 μM of CcO in 5 mM Tris buffer, pH 7.4 (low salt condition) or 5 mM Tris buffer with 100 mM KCl, pH 7.4 (high salt condition) was centrifuged in a centricon-100, which retains CcO but allows free

cytochrome c to pass the membrane, for 10 min at 3°C. After the centrifugation, the concentration of cytochrome c in the filtrate and the original solution together with that of CcO in the original solution were determined with UV-Visible spectra.

Finally, to determine the effect of non-covalent ATP binding to cytochrome c on the binding constant between native cytochrome c with CcO at low ionic strength, 30 or 300 μM of ATP was also present in some of the low salt experiments with native cytochrome c.

Since the filtrate volume was about 20 % of the total volume, the species separated was assumed to reflect the concentration of the species in equilibrium in the original solution. Therefore, in experiments on the binding of cytochrome c or its ATP(ADP) adducts, we have

$= C_S^F$

$= C_S^O - C_S^F$

$= C_E^O - [E \cdot S]$

in which [S] is the concentration of free substrate (cytochrome c or its ATP(ADP) adduct), [E] is the concentration of free enzyme (CcO) and [E·S] is the concentration of the enzyme substrate complex. C_S^F and C_S^O denote the concentration of substrate in the filtrate and original solution respectively, C_E^O is the concentration of enzyme in the original solution. Similar relationship may be employed for the determination of the binding of ATP to cytochrome c.

Steady-state kinetics of CcO with native and 8-Azido-ATP (-ADP) modified cytochrome c. The steady-state turnover rate of CcO was determined by the polarographic method. 1 to 16 μM of native or 8-azido ATP modified cytochrome c was added to 5 nM of oxidase in 5 mM Tris, 100 mM KCl, 5 mM ascorbate, 0.1% lauryl-D-maltoside, 0.7 mM TMPD, pH 7.4, and the turnover rate was monitored as the oxygen consumption rate with a VSI Model 53 Oxygen Electrode (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio). The data are presented in Eadie-

Hofstee plots. The activity is expressed as molecular turnover (TN = mole cytochrome c/sec mole cytochrome c oxidase) at 20°C.

Transient electron transfer from cytochrome c to CcO. Transient absorbance data were taken with an excimer dye laser at the Laser Facility of the Beckman Institute, California Institute of Technology. Electron transfer was initiated by the laser flash photolysis technique developed by Hazzard *et al.* (Hazzard *et al.*, 1991). When 5-deazariboflavin (5-DRF) is excited by a laser flash, it abstracts an electron from EDTA and becomes a flavin semiquinone. The flavin semiquinone rapidly reduces cytochrome c, which subsequently reduces CcO at a slower rate. The reoxidation of reduced cytochrome c by the oxidase was followed by monitoring the 550 nm absorption peak of the cytochrome c. The electron transfer rate was also followed by monitoring the reduction of cytochrome a of CcO at 604 nm. These experiments were performed on a solution containing 10 μM cytochrome c and 5-25 μM of oxidase in a buffer of 5 mM Tris containing 1 mM EDTA, 0.1% lauryl maltoside, 33 μM 5-DRF, and 100 mM KCl, pH 7.4. The sample cuvette was sealed and degassed before being subjected to the excimer dye laser flash (BPBD at 395). The optical signals represented the accumulation of 30 flashes.

Redox potential of cytochrome c and the 8-Azido-ATP adducts. The redox potential of cytochrome c and its 8-azido-ATP adducts were measured by the method of Wallace *et al.* (Wallace *et al.*, 1986) by monitoring the redox equilibrium

Ferricyanide + Ferrocytochrome c <==> Ferrocyanide + Ferricytochrome c.

RESULTS:

Modification of cytochrome c with 8-azido ATP. Upon modification of cytochrome c with 8-azido ATP, the binding of adduct to the CM cellulose column is reduced and the adducts are eluted out from column ahead of unmodified cytochrome c. The ATP-cytochrome c adducts (fraction 1 and 2)

show increased absorption in the UV region as previously reported by Craig and Wallace (1993). The stoichiometry of the modification of the cytochrome c by 8-azido ATP was determined by MALDI Time-of-flight mass spectroscopy (Table1). The mass spectra of fraction 1 of the modified cytochrome c showed a peak at 12,870 amu and a minor peak at 12,478 amu; fraction 2 of the modified cytochrome c showed a peak at 12,860 amu and another peak at 12,372 amu. Native cytochrome c showed a single mass peak at 12,344 amu. The mass peaks at 12,870 amu and 12,860 amu of fraction 1 and 2 correspond to single modification by 8-azido ATP. The minor mass peaks at 12,478 amu, and 12,372 amu from fraction 1 and 2 most likely correspond to modified cytochrome c's, in which the ATP moiety has been dissociated from the adducts during sample manipulation. Peptide mapping of these cross-linked products, by cyanogen bromide fragmentation followed by HPLC separation or by the method of Brautigan *et al.* (1978), failed to reveal the site of cross linking due to the instability or lability of the cross-linked adducts. The ATP moiety was hydrolyzed from the peptide during the manipulation of the peptide as determined by the mass spectra of the fragments. Although we could not discern the chemical difference between fraction 1 and 2 of the ATP-cytochrome c adducts, the two fractions most likely represent only differences in the site of ATP crosslinking, as evidenced by the similarities in the binding of the adducts in the two fractions to CcO as well as their kinetic behaviors.

The effect of 8-azido ATP (ADP) modification on the binding of ATP to cytochrome c and cytochrome c binding to CcO. The affinity of ATP binding to native and 8-azido ATP (ADP)-modified cytochrome c at low ionic strengths were determined by the fast filtration method following equilibration of 30 μM of ATP with 10 μM of cytochrome c or its ATP (ADP) adducts (Table 2). Upon 8-azido ATP modification, we observe a dramatic decrease in the ability of cytochrome c to bind ATP. A decrease is also observed upon 8-azido ADP modification though the effect is less significant.

Table 1. Molecular mass (a.m.u.) of native and 8-azido ATP modified cytochrome c[a]

Samples	Molecular mass	
	Peak 1(major)	Peak 2(minor)
fraction 1 of 8-azido-ATP modified cytochrome c	12,870	12,478
fraction 2 of 8-azido-ATP modified cytochrome c	12,860	12,372
native cytochrome c	12,344	

[a] the molecular mass of ATP^{-4} is 503 amu.

Table 2. Apparent binding stoichiometry of ATP to native and 8-azido ATP (ADP) modified cytochrome c at low ionic strength (5 mM)

Samples	Apparent binding Stoichiometry ([ATP]:[cytochrome c])
native cytochrome c	1.1
fraction 1 of 8-azido-ATP modified cytochrome c	0.3
fraction 2 of 8-azido-ATP modified cytochrome c	0.4
8-azido-ATP modified cytochrome c	0.9

The effects of 8-azido ATP (ADP) modification on the binding of cytochrome c to CcO are summarized in Table 3. For native cytochrome c binding to CcO, it is well known that the redox protein partners form a very strong complex at low ionic strengths. While the 8-azido ADP-modification has a relatively small effect on the binding between cytochrome c and CcO under these conditions, 8-azido ATP modification (fraction 1 and 2) reveals dramatic effects on the binding constant (3 orders of magnitude lower than in the case of native protein). However, this effect is greatly reduced as the solution ionic strength is increased to physiological values. In fact, the binding constants between the ATP-cytochrome c adducts and CcO are almost unchanged with ionic strengths compared to that of native cytochrome c.

Taking advantage of the strong binding of ATP to cytochrome c (K_d between ATP and cytochrome c in 10 mM Tris-cocodylate buffer was determined to be 40 μM; Craig and Wallace 1991) as well as cytochrome c to CcO at low ionic strengths, we have attempted to determine the effect of free ATP on the binding constant between cytochrome c and CcO under low ionic strengths. These results are also included in Table 3. It is noteworthy that, although ATP should saturate its binding site on cytochrome c under these conditions (at least when the concentration of ATP is 300 μM), the effect of this ATP binding on the affinity of cytochrome c to CcO is very small compared to the effects of covalent 8-azido ATP modification of the cytochrome c.

Steady-state turnover of CcO with native and 8-azido-ATP (-ADP) modified cytochrome c. The results of steady-state kinetics of CcO with the native and modified cytochrome c are depicted in Figure IV.1. Since the concentration range of cytochrome c used correspond to the low affinity kinetic phase, we obtained only linear relations in the Eadie-Hofstee plot. Linear fitting of the kinetic curves yielded K_m's and V_{max}'s for the reaction of native and ATP-cytochrome c adducts with CcO. As shown in Table 4, the steady-state turnover of the CcO was significantly slower in the case of the cytochrome c-ATP adducts. The adducts from fractions 1 and 2 exhibited only 14% and 15% of the V_{max} of the native protein, respectively. These results are in agreement with

Table 3. Binding affinity of native, and 8-azido-ATP (-ADP) modified cytochrome c to CcO under low and high ionic strengths

Samples	K_a (at low salt)[a] (μM^{-1})	K_a (at high salt)[b] (μM^{-1})
native cytochrome c	243.0	0.039
fraction 1 of 8-azido-ATP modified cytochrome c	0.146	0.015
fraction 2 of 8-azido-ATP modified cytochrome c	0.067	0.023
8-azido-ADP modified cytochrome c	37.2	
native cytochrome c in the presence of 30 μM ATP	108.3	
native cytochrome c in the presence of 300 μM ATP	39.2	

[a] Ionic strength is 5 mM
[b] Ionic strength is 105 mM

Figure IV.1. Eadie-Hofstee plots of the steady-state kinetics of native and 8-azido ATP (-ADP) modified cytochrome c with CcO. The activity of CcO in the presence of native and 8-azido ATP (-ADP)-modified cytochrome c was assayed as detailed under Methods. (A) native; (B) 8-azido ADP modified; (C) fraction 1 of 8-azido ATP modified; and (D) fraction 2 of 8-azido ATP modified cytochrome c.

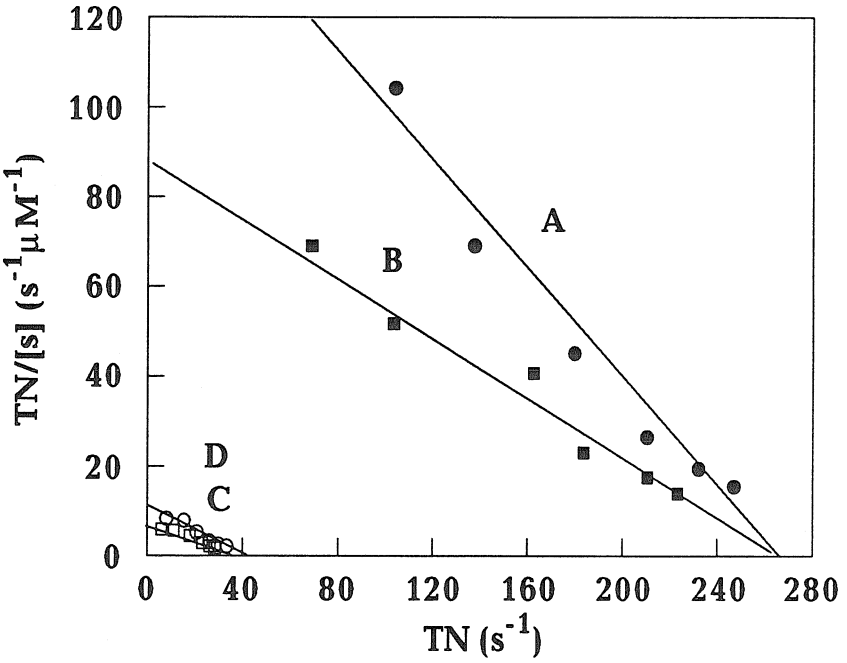

120
100
80
60
40
20
0
0
40
80
120
160
200
240
280
TN/[s] ($s^{-1}\mu M^{-1}$)
TN (s^{-1})
A
B
C
D

Table 4. Kinetic parameters derived from the polarographic assays

Samples	K_m (μM)	V_{max}
native cytochrome c (low affinity phase)	1.65	263
fraction 1 of 8-azido-ATP modified cytochrome c	5.00	38
fraction 2 of 8-azido-ATP modified cytochrome c	3.61	40
8-azido-ADP modified cytochrome c	2.86	263

Craig and Wallace (1993). In addition, the ATP-cytochrome c adducts exhibit a higher K_m (lower affinity) than the native enzyme. On the other hand, the ADP-cytochrome c adduct shows an increased K_m, but the V_{max} is unchanged compared to that for native enzyme.

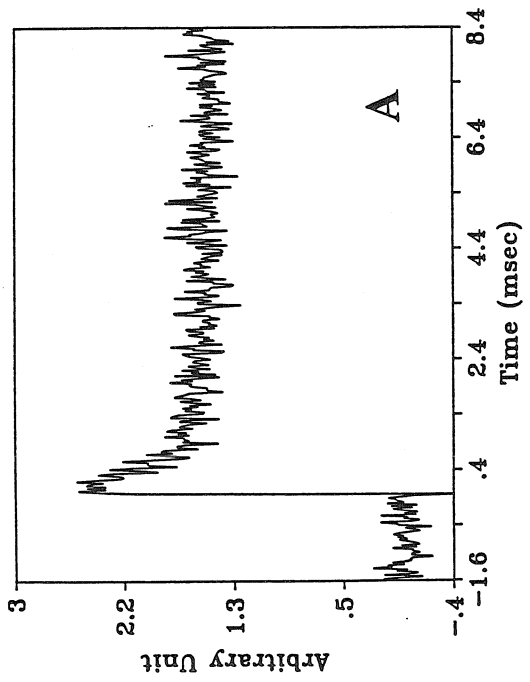
Transient absorption study of the electron input from native and 8-azido-ATP modified cytochrome c to CcO. Figure IV.2A and B shows a typical transient kinetic trace observed for the intracomplex electron transfer between horse cytochrome c and fully oxidized native bovine CcO at 1:1 mole ratio and 110 mM ionic strength. The reduction of ferricytochrome c by 5-DRF semiquinone and its subsequent reoxidation by CcO were monitored at 550 nm (Figure IV.2A). The kinetic trace corresponding to the cytochrome c reoxidation is biphasic and fits well to a sum of two exponential, as noted earlier (Pan *et al.*, 1991). An observed kinetic constant of 941 s^{-1} is obtained for the fast phase. The reduction of cytochrome a in the CcO was followed at 604 nm (Figure IV.2B). A rate constant of 897 s^{-1} was obtained for the fast phase.

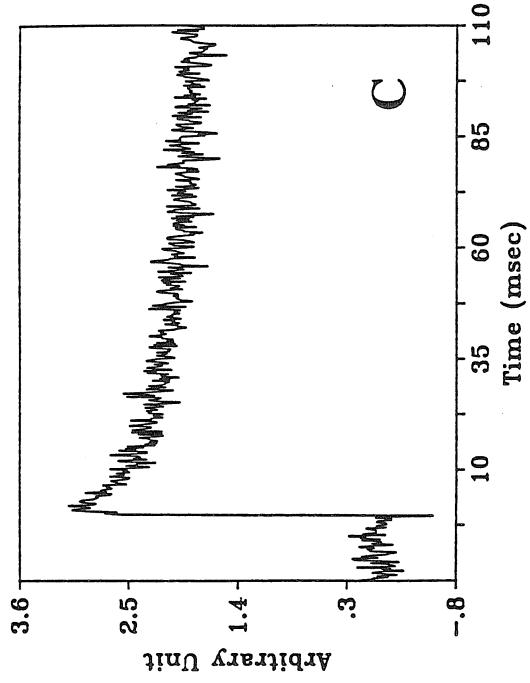
Under otherwise identical conditions, the two fractions of the cytochrome c-ATP adduct display significant slower electron transfer kinetics (see Figure IV.2C and D). Figure IV.2C shows the reoxidation of cytochrome c and Figure IV.2D shows the reduction of cytochrome a obtained with fraction 1 of the cytochrome c-ATP adduct. A kinetic constant of 36 s^{-1} is observed for both the reoxidation of the cytochrome c-ATP adduct and the reduction of cytochrome a. Similar results were obtained with the ATP-adduct of fraction 2 (kinetic constant 30 s^{-1}).

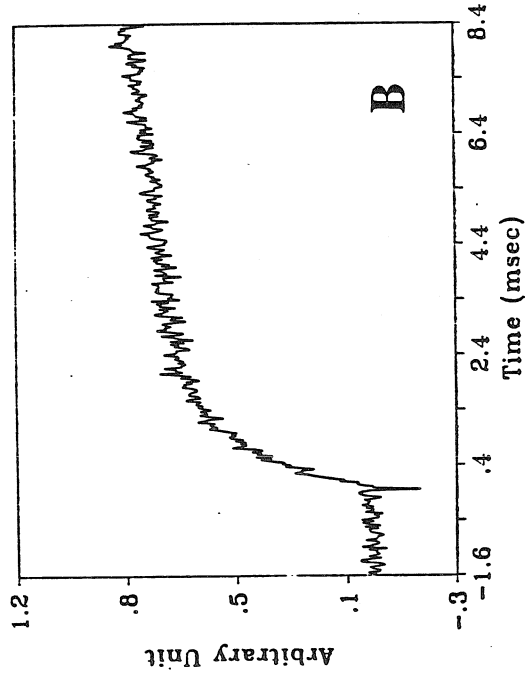
The above experiments have been repeated for various oxidase concentrations under otherwise identical conditions including the ionic strengths. From the kinetic constants observed at different oxidase concentrations, we have determined the association constant for formation of the ferrocytochrome c: CcO complex and the first-order rate constant for the intracomplex electron transfer (Pan *et al.*, 1991). The equation for fitting the kinetic data is

$$k_{obsd} = k_{et} K_a [CcO]_{ox} / (K_a[CcO]_{ox} + 1) \quad (1)$$

Figure IV.2. Intracomplex electron transfer to native CcO from ferrocytochrome c, and one of its 8-azido-ATP modified adducts. (A): Reduction of cytochrome c by photogenerated flavin semiquinone and reoxidation of ferrocytochrome c followed at 550 nm. (B): Reduction of cytochrome a by the ferrocytochrome c observed at 604 nm. (C): Reduction of the 8-azido-ATP modified cytochrome c (fraction 1) reduced by photogenerated flavin semiquinone and reoxidation of ferrocytochrome c-ATP adduct followed at 550 mm. (D): Reduction of cytochrome a by the ferrocytochrome c-ATP adduct followed at 604 nm. These experiments were performed on a solution containing 10 μM cytochrome c and 10 μM of oxidase in a buffer of 5 mM Tris containing 1 mM EDTA, 0.1% lauryl maltoside, 33 μM 5-DRF, 100 mM KCl, pH 7.4.


A
Arbitrary Unit
3
2.2
1.3
.5
-.4
-1.6
.4
2.4
4.4
6.4
8.4
Time (msec)


C
Arbitrary Unit
3.6
2.5
1.4
.3
-.8
10
35
60
85
110
Time (msec)


B
Arbitrary Unit
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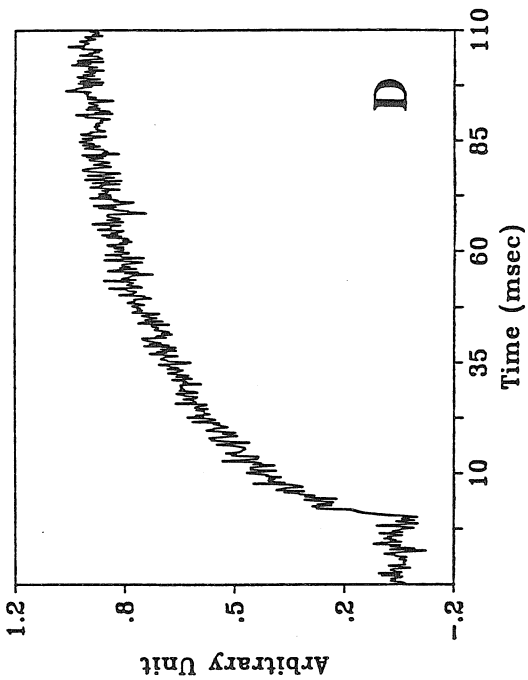
.8
.5
.1
-.3
-1.6
.4
2.4
4.4
6.4
8.4
Time (msec)


D
Arbitrary Unit
1.2
.8
.5
.2
-.2
10
35
60
85
110
Time (msec)

where K_a is the association constant for the formation of the ferrocytochrome c:CcO complex and k_{et} is the intracomplex electron transfer rate constant. When the concentration of CcO is sufficiently high,

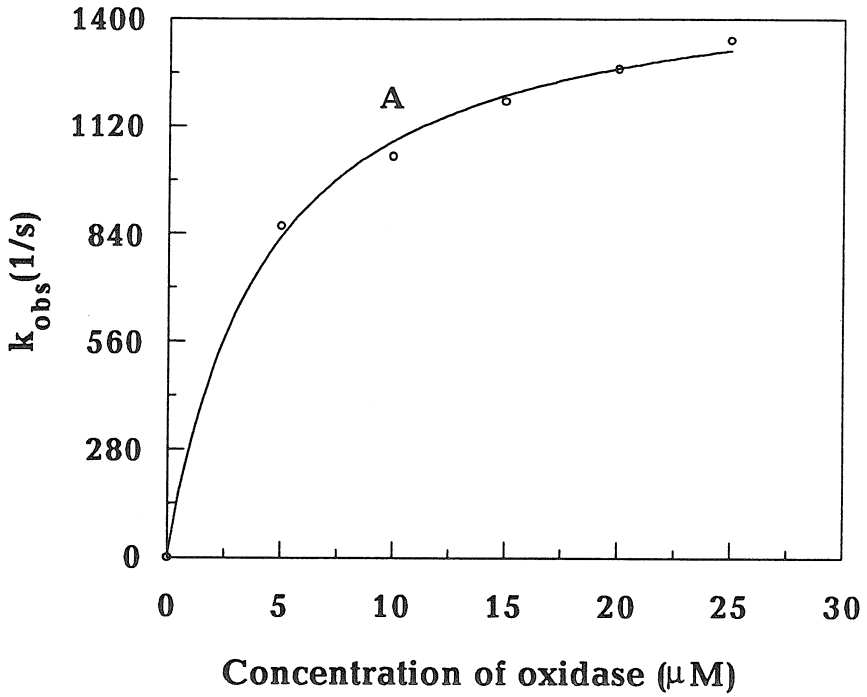
$$k_{obsd} = k_{et} \quad (2)$$

so that the maximal observed rate equals the intracomplex electron transfer rate.

The best fits of the kinetic data obtained for native cytochrome c and the ATP-adducts (both fraction 1 and 2) to equation (1) are shown in Figure IV.3 and the results are tabulated in Table 2. The effect of ATP-modification on the binding of cytochrome c to the oxidase is small: K_a is reduced by 24 % in the case of fraction 1 and 70 % for fraction 2. On the other hand, the intracomplex electron transfer rate constant k_{et} is dramatically reduced for the ATP-adducts (Table 5). Compared to native cytochrome c, k_{et} has been reduced to 3.9 % and 4.5 % for the two adducts, respectively. It should be noted that k_{et} for the native cytochrome c (1546 s^{-1}) obtained in this study is lower than that observed in our previous study (2580 s^{-1}) (Pan *et al.* 1991). Since, the intracomplex electron transfer rate constant does vary from batch to batch of enzyme, it could be that the activity of the oxidase in the batch used in the present study is lower. Our present value of k_{et} is close to that previously reported by Hazzard *et al.* (1470 s^{-1}) (Hazzard *et al.*, 1991).

The redox potentials of native and 8-Azido-ATP modified cytochrome c. In order to ascertain whether the slower k_{et} observed for the cytochrome c-adduct could be due to a change in the redox potential of the cytochrome c brought about by the ATP-modification, we have measured the redox potentials of the adducts. According to the method of Wallace *et al.* (Wallace *et al.*,1986), log([ferrocyanide]/[ferricyanide]) has been plotted against log([ferrocytochrome c]/[ferricytochrome c]) for native cytochrome c and the two ATP-adducts. Extrapolation of the data to [ferrocytochrome c]/ [ferricytochrome c] = 1 allows determination of the redox potential for the heme using the expression $E = E^{o'} - 0.059 \log([\text{ferrocyanide}] / [\text{ferricyanide}])$. The value of $E^{o'}$ for the ferrocyanide/ferricyanide couple is taken to be +0.43 volts.

Figure IV.3. Kinetics of electron transfer between ferrocytochrome c (10 μM) and CcO at various CcO concentrations (5-25 μM) and an ionic strength of 110 mM. The concentration of cytochrome c remains unchanged as the concentration of CcO is varied. The reaction conditions are described in experimental section. 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 CcO. The solid curves represent the best fits of the data to equation 3. (A): Native cytochrome c. (B): Fraction 1 of 8-azido-ATP modified cytochrome c. (C): Fraction 2 of 8-azido-ATP modified cytochrome c. The experimental conditions are identical with those in FIG. 2.


1400
1120
840
560
280
0
A
k_{obs}(1/s)
0
5
10
15
20
25
30
Concentration of oxidase (μM)

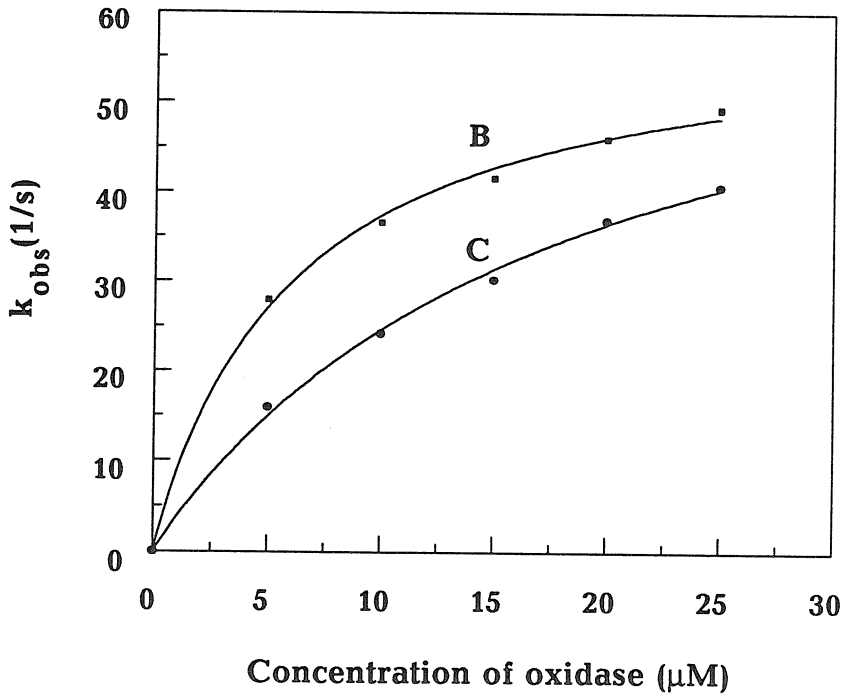

60
50
40
30
20
10
0
B
C
k_{obs}(1/s)
0
5
10
15
20
25
30
Concentration of oxidase (μM)

Table 5. Kinetic parameters for intracomplex ET between cytochrome c and cytochrome c oxidase

Sample	K_a [x 10^{-4} (M^{-1})]	k_{et} (s^{-1})
native cytochrome c	4.6	1550
modified cytochrome c fraction 1	3.2	60
modified cytochrome c fraction 2	1.1	70

For the native cytochrome c, and fraction 1 and fraction 2 of the ATP-adduct, the redox-potentials are 265, 265 and 262 mV, respectively. Thus, there is no significant change in the redox potential among these different forms of cytochrome c. Accordingly, the retardation of the electron transfer rate in the case of the 8-azido-ATP adducts could not be due to a change in the driving force between the c-heme and Cu_A (and/or cytochrome a).

DISCUSSION:

8-azido ATP, upon illumination with UV light, cross-links to cytochrome c, forming cytochrome c-ATP adducts. The site of cross-linking has not been determined. Peptide mapping of the ATP labeled amino acid has failed, due to the lability or instability of the adducts. The ATP moiety dissociates from the cytochrome c in the process of manipulation. The dissociation of ATP from cytochrome c has been reported by Craig and Wallace (1993), in their attempts to fragment the ATP-cytochrome c adducts with cyanogen bromide for separation. In earlier work, Montecucco *et al.* (1986) also failed to observe the association of 8-azido [γ-P^{32}] ATP with the cytochrome c after cross-linking with UV lamp in the presence of CcO following manipulation of the sample. Thus, it appears that the adduct(s) between 8-azido-ATP and cytochrome c are relatively labile or unstable, and the covalent linkage is readily broken upon manipulation of the adduct(s). This suggests that the ATP moiety does not form adducts with the cytochrome c through formation of a carbon-nitrogen bond or via ring rearrangement (Knowles, 1972).

Upon formation of the ATP adducts, cytochrome c loses some of its ability to bind ATP. Data from fast filtration, steady-state kinetics, and transient electron transfer experiments demonstrate that the binding constant between the ATP-cytochrome c adducts and CcO decreases by a factor of about two relative to the native cytochrome c at physiological ionic strength. Under conditions of low ionic strengths, the effect of the 8-azido-ATP modification on the binding constant of cytochrome c to CcO is more dramatic. From these data, we reason that charge interactions

(especially salt bridge formation) between the positive charges of cytochrome c (lysine's) and the negative charges of CcO (carboxylate's) are important contributing factors to the binding between the redox protein partners under low ionic strengths. The modification of cytochrome c with 8-azido ATP must greatly influence some of the residues involved in the formation of the salt bridge(s) between the proteins. These charge interactions, of course, contribute less to the binding at high ionic strengths, so does the chemical modification. Support of these arguments come from crystallographic studies of the structure of cytochrome c peroxidase (CcP) and cytochrome c complexes (Pelletier and Kraut, 1992), where it has been shown that CcP:Cc(Horse) grown at low ionic strengths exhibits closer distances between potential charge-mediated hydrogen bonds atoms than CcP:Cc(Yeast), grown under physiological ionic strengths.

One interesting observation of the binding between ATP-cytochrome c adduct(s) and CcO is that, unlike that in the case of native cytochrome c, the binding constant is not greatly influenced by the solution ionic strength. This could only happen when the charge interaction between cytochrome c and CcO contributes in a minor way to the binding. This is quite unusual for cytochrome c binding to its redox protein partner. Accordingly, the docking of ATP-cytochrome c adducts to CcO could be quite different from that of native cytochrome c, where the charge interactions dominate the details of the docking. In support of this conclusion, we find that the binding of free ATP, at concentrations beyond that required to saturate with cytochrome c at low ionic strengths, does not show as dramatic an effect on the binding of 8-azido ATP modified cytochrome c to CcO, although it does decrease the binding affinity. The implication is that there is a difference in the docking of cytochrome c with CcO in the presence of noncovalent ATP binding and upon cross-linking by 8-azido ATP.

It is now generally agreed that Cu_A is the primary electron acceptor in CcO, although electron can also be transferred from heme c of cytochrome c directly to heme a, albeit with reduced rate (Pan *et al.*, 1991). From Cu_A, the electron can then be transferred to heme a of CcO. Under physiological

ionic strengths, the electron transfer rate from heme c to heme a is reported to be from 1,500 to 2,500 s^{-1} (Pan *et al.*, 1991, 1993; Hazzard *et al.*, 1991; Larsen *et al.*, 1992). The electron transfer from Cu_A to heme a has been determined to be around $2x10^4$ s^{-1} (Pan *et al.*, 1993; Morgan *et al.*, 1989; Kobayashi *et al.*, 1989). Since the electron transfer from Cu_A to heme a is faster than that of direct transfer from heme c to Cu_A, the observed electron transfer rate from heme c to heme a is limited by that from heme c to Cu_A. Under low ionic strength conditions, in which cytochrome c forms a tight complex with CcO, the electron transfer from ruthenated cytochrome c to CcO was shown to be faster than 10^5 s^{-1} while the electron transfer rate from Cu_A to heme a remains unchanged (Pan *et al.*, 1993). With 5-DRF as electron donor, however, the electron transfer rate falls off as the ionic strength is decreased from the physiological values. One possible explanation for the different behavior between the two electron donating systems under low ionic strength conditions could be the different pathway of electron transfer from the primary donor to heme c. In the case of 5-DRF, as suggested by Pan *et al.* (1993), the electron may have to be donated to heme c through encounters with cytochrome c at certain surface domains, which are sequestered by CcO upon formation of the tight "fast electron transfer" complex under low ionic strengths. If this is the case, electron input from the 5-DRF to cytochrome c, could be limited to those cytochrome c molecules that bind to CcO with the "slow electron transfer" orientation with the surface domains available for electron input from 5-DRF. Such is not the case in the ruthenated cytochrome c injection system, since the electron is donated from the ruthenium to heme c via covalent bonds. As the ionic strength is increased to physiological values, most of the cytochrome c is dissociated from the CcO, so the details of the electron input into the cytochrome c no longer become an issue. Although there are still some variations in the electron input rate from cytochrome c to CcO at low ionic strengths with different electron injection systems, it is generally agreed that the ionic strengths exert effects on the electron transfer by altering the docking between the redox protein partners (Pan *et al.*, 1993; Hazzard *et al.*, 1991).

The docking surface of cytochrome c to CcO have been studied by Ferguson-Miller *et al.* (1978) by the chemical modification method. Lysine's 8, 13, 72, 87 have been demonstrated to be important in the docking of cytochrome c to CcO. Upon modification of these lysine's, dramatic changes on the binding constant and V_{max} have been observed. The docking surface predicted by the above studies have been shown to be almost identical to that of the docking to cytochrome c peroxidase as revealed by the recent crystal structure of the CcP: Cc complex (Pelletier and Kraut, 1992). All the lysine's on cytochrome c predicted to be important to the binding to CcO are also involved in the docking to CcP to various extents.

Although our attempts to locate the site of 8-azido ATP modification by peptide mapping of ATP-cytochrome c adduct were not successful due to the lability/instability of the adducts, it is well known that modification of all the lysine groups in cytochrome c greatly decreases the yield of the 8-azido ATP modification beyond what could be accounted for by the site occupancy alone. This may implicate the lysines in the modification reaction (Craig and Wallace, 1993). In the structure of cytochrome c, lysine's 86, 87, 88 are located close to arginine 91, which is the ATP binding site on cytochrome c. Thus, one of these lysine's could become modified by 8-azido ATP.

The modification of cytochrome c with 8-azido ATP interferes significantly with the docking of cytochrome c to CcO according to our binding studies, but not the electrostatic binding of free ATP to cytochrome c. It is evident that certain residue(s) (possibly lysine 86 or 87) on cytochrome c, which are important to the docking to CcO, has been modified, distinct from electrostatic ATP binding site(s). Based on this reasoning, we surmise that ATP-cytochrome c adducts assumes different docking conformations with CcO from that of native cytochrome c. The insensitivity to the solution ionic strength of the association of ATP-cytochrome c adducts with CcO supports this contention.

The change of the docking conformation of ATP-cytochrome c adducts to CcO from that in the case of native cytochrome c could easily result in a decrease of electron transfer rate by changing the electronic coupling between heme c and Cu_A. Currently, there are two different opinions on how the intervening medium could influence the electron coupling between an electron donor and an acceptor. According to one group of proponents (Moser *et al.*, 1992), the electron transfer rate decreases exponentially with the distance between the donor and acceptor regardless of the intervening medium. On the other hand, Gray and coworkers (Beratan *et al.*, 1991; Wuttke *et al.*, 1992) assert that electron transfer rate depends not only on the distance between donor and acceptor but also on the intervening medium (or the pathway(s)). Although an explanation for these two different opinions have been given (Evenson and Karplus, 1993), it remains to be clarified how nature designs its electron transfer processes. Our experimental observations of the retardation of electron transfer from ATP-cytochrome c adducts to CcO could be explained by either an increase in distance between heme c edge and Cu_A (exponential model), or alternatively by an altered electron transfer pathway(s) (non-exponential model), with different docking conformations of the ATP-cytochrome c adducts from that of native cytochrome c in the Cc:CcO complex.

Although the electron input rate from the ATP-cytochrome c adducts to CcO is less than 5 % of that in the case of native cytochrome c, the corresponding difference in the steady-state kinetic rate is less dramatic. This could be interpreted in terms of different rate limiting steps in the two experiments. In the steady-state experiments, the rate limited step may or may not be the electron input from cytochrome c to CcO. This is probably not the case for cytochrome c. With the ATP-cytochrome c adducts, the steady-state turnover is clearly limited by the electron input from the heme c.

The feedback control of the respiratory chain by ATP is an important issue for the bioenergitic of a cell. A few studies have contributed to our understanding of this feedback control. Most of the studies have focused on the ATP binding of ATP to CcO and its inhibition of the respiration chain

(Montecucco *et al.*, 1986; Rigoulet *et al.*, 1987; Bisson *et al.*, 1987; Malatesta *et al.*, 1987; Huther and Kadenbach 1986, 1987, 1988; Antonini *et al.*, 1988; Reimann and Kadenbach, 1992). However, in a recent study by Craig and Wallace (1993), sensitivity of the respiratory chain toward the energetic state of the cell was attributed to the direct binding of ATP to cytochrome c and the consequence of this binding on inhibition of the respiration. The efficacy of cytochrome c-ATP adducts in restoring dioxygen consumption in cytochrome c-depleted mitochrondria relative to native cytochrome c, reported recently by Craig and Wallace (1993), are consistent with our present experimental finding. Thus, ATP appears to be capable of influencing the kinetics of cytochrome c-CcO complex on both the cytosol and matrix sides of the inner mitochondria membrane (Huther and Kadenbach 1987, 1988), albeit with different characteristics. Nevertheless, It is unlikely that the influence of ATP on the kinetics of intracomplex electron transfer could be solely attributed to the interaction of ATP with cytochrome c. Although the ATP-cytochrome c adducts could have deleterious effects on the cytochrome c/CcO electron transfer kinetics, free ATP itself does not exhibit the same effect on either binding of cytochrome c to CcO or the electron input rate (Lin *et al.*, unpublished data) as the ATP-cytochrome c adducts. The significant retardation of the electron input from ATP-cytochrome c adducts is more likely due to the modification of some amino acid which is important in the docking of cytochrome c. It remains to be determined whether the binding of free ATP has similar effects on the electron transfer from cytochrome c to CcO and why nature designs a binding site for ATP on cytochrome c. Interestingly, we do observe some decrease in the binding affinity of cytochrome c to CcO upon the binding of free ATP under conditions of low ionic strengths.

In conclusion, the cytochrome c-ATP adducts show lower binding affinity and significantly slower electron transfer rate to CcO. The chemical modification does not alter the redox potential of the heme c. The retardation of the electron transfer rate is possibly due to the perturbation of the docking between cytochrome c and CcO resulting in changes in the electronic coupling between heme c and Cu_A (and cytochrome a). However, it is unclear whether noncovalent electrostatic ATP

binding to cytochrome c will exhibit any effects on the electron transfer, although the specifics certainly will be different from those of the cytochrome c-ATP adducts according to binding studies.

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Chapter V

Electron transfer from cytochrome c to 8-azido-ATP-modified cytochrome c oxidase

ABSTRACT:

Bovine heart cytochrome c oxidase (CcO) has been modified by 8-azido-adenosine 5'-triphosphate (8-azido-ATP) and the electron transfer activity from ferrocytochrome c to the modified CcO under physiological ionic strengths has been studied by the laser flashphotolysis technique with 5-deazariboflavin and EDTA as the electron donor. The kinetics of intermolecular electron transfer between the redox protein partners was shown to be reduced significantly. In addition, there is a significant decrease in the binding affinity of the cytochrome c to the oxidase upon 8-azido-ATP modification. The 8-azido-ATP-modified CcO exhibited 69% (50 %) of the intracomplex electron transfer rate (k_{et}) and 65 % (56 %) of the association constant (K_a) normally observed between cytochrome c and native CcO under otherwise identical conditions. Since the effective electron transfer rate constant is the product of k_{et} and K_a under non-saturation conditions, the overall electron transfer rate has been curtailed by over a factor of 2. Similar observations have been noted with the native CcO in the presence of 3 mM ATP. In contrast, the redox-potential of neither Cu_A nor cytochrome a was altered upon 8-azido-ATP modification or in the presence of 3 mM ATP. Also, no gross structural changes at both the Cu_A or cytochrome a sites were noted, as evidenced by lack of any spectral perturbations in the EPR signals from both of these centers. We conclude that ATP modulates the electron transfer from cytochrome c to CcO by interacting with the CcO and altering allosterically the docking. In this manner, ATP can affect the branching of the electron input from ferrocytochrome c to cytochrome a and Cu_A.

INTRODUCTION:

Cytochrome c oxidase (CcO), the terminal component of the mitochondrial respiratory chain, catalyzes the transfer of electrons from cytochrome c to dioxygen, and couples the electron transfer to the active transport of protons across the mitochondrial inner membrane. The steady-state reduction of cytochrome c catalyzed by this enzyme shows distinctive biphasic behavior (Nicholls, 1964; and Ferguson-Miller *et al.*, 1976). The V_{max}'s for the high and low affinity phases are 10-40 s^{-1} and 100-200 s^{-1}, respectively (Errede and Kamen, 1978; Rosevear *et al.*, 1980). Another interesting feature of the CcO-catalyzed reaction is that ATP, under physiological concentrations, influences the kinetics by abolishing, at least to a greater extent, the high affinity phase; in addition, V_{max} of the low affinity phase is reduced (Ferguson-Miller *et al.*, 1976). The latter effect is relevant to the control of turnover of the enzyme under physiological conditions since the rates fall within the physiological range.

Over the years, there has been much evidence accumulating to indicate that ATP regulates cellular respiration, especially the terminal electron transport steps from cytochrome c to dioxygen mediated by CcO. Kadenbach and coworker (Huther and Kadenbach, 1986, 1987, 1988) have proposed that the control is allosteric, with ATP-binding to the oxidase modulating the details of the enzyme turnover, including the intramolecular electron transfer rates and the efficiency of biological energy transduction. Montecucco *et al.*, (1986) have observed labeling of subunit IV and VII upon photocrosslinking of CcO with 8-azido-[γ-^{32}P]ATP. These workers have suggested that subunit IV and one of the subunit VII peptides provide the binding loci for the ATP. If so, this binding appears to exert a long-range conformational change in the structure of CcO, which affects the tertiary folding of subunit II and its efficacy to interact with cytochrome c (Bisson *et al*, 1987). Apparently, the negatively charged phosphate moiety of the ATP, more than the heterocyclic base linked to the sugar moiety, is the determinant here, since UTP has a similar kinetic effect on the oxidase activity as ATP (Bisson *et al.*, 1987).

Several groups have attempted to mimic the specific effects of ATP on the steady-state kinetics of CcO-catalyzed reaction by using covalently modified 8-azido-ATP CcO in the kinetics studies. The advantage of this approach is that the nonspecific effects of ionic strength on the docking of the redox protein partners can be obviated, particularly at high ATP concentrations. Free ATP also binds to cytochrome c under sufficiently high ATP concentrations and this phenomenon may have deleterious effects on the interaction between cytochrome c and CcO (Craig and Wallace, 1991, 1993). Similar kinetic effects have been observed for 8-azido-ATP-modified CcO as for CcO in the presence of moderate ATP concentrations (Huther and Kadenbach, 1986).

Although there are ample data to support the regulatory effects of ATP on the activity of CcO, our understanding of the problem is still rudimentary. In this paper, we attempt to study the effects of ATP binding to CcO on the kinetics of the input of the first electron from cytochrome c to CcO. Following earlier work, we have exploited the use of 8-azido-ATP-modified enzyme in these experiments. Recent advances in transient spectroscopy have provided us with a very powerful tool to examine electron transfer processes in biological systems. We find that 8-azido-ATP modification of CcO leads to lower binding affinity for the cytochrome c as well as slower electron transfer from ferrocytochrome c to the oxidase. Since ATP-binding or 8-azido-ATP modification shows no observable change on the redox potential of Cu_A or cytochrome a as well as the EPR spectra of the two low potential centers, the observed effects of ATP on the activity of the oxidase could only be due to changes in the docking between the cytochrome c and the CcO, changes in either the binding affinity and/or electron transfer pathways.

MATERIALS AND METHODS:

Material. CcO was isolated and purified from bovine heart mitochondria according to the method of Hartzell and Beinert (1974). The enzyme preparation was stored at -78°C before use. Enzyme

concentrations were determined from the absorbance change $\Delta A_{red\text{-}ox}$ at 605 nm using an extinction coefficient of 24 mM^{-1} (Van Gelder, 1966). Horse heart cytochrome c (type VI), DEAE Cellulose ionic exchange gel, ATP (grade II) and 8-Azido-ATP were obtained from Sigma Chemical Co. (St. Louis, MO). Other chemicals were of the highest grade available.

Modification of CcO with 8-azido-ATP or 8-azido-ADP. CcO was diluted to a final concentration of 15 μM in 10 mM Hepes buffer, pH 7.4, 0.1 % lauryl-D-maltoside. 3 mM of 8-azido-ATP or 8-azido-ADP was added to the samples and incubated for 20 min at 4°C. The mixture was then illuminated with a Mighty Bright UV source (Hoefer Scientific instrument, San Francisco) in a 20 ml vial for one hour. To remove the unreacted 8-azido-ATP, ADP and reaction side products, the samples were loaded onto a DEAE-cellulose column and eluted with a linear gradient of 0-100 mM of NaCl with 5 mM phosphate and 0.1 % lauryl-D-maltoside buffer, pH 7.4. The CcO fraction was collected from the column, and then washed with 5 mM Tris buffer containing 100 mM KCl, 0.1 % lauryl-D-maltoside, pH 7.0 in Centricon 100 miniconcentrators. The modified CcO was stored at -78°C until use.

Preparation of Cu_A-depleted CcO. Cu_A-depleted CcO was prepared according to the method of Pan *et al.* (1991). Cu_A was first converted to a type II center by pHMB treatment (Gelles and Chan, 1985). The copper ion was then removed from the modified Cu site by dialysis of the sample against EDTA (Li *et al.*, 1987).

Determination of the extent of 8-azido-ATP modification of CcO from TNP-ATP-binding to native and 8-azido-ATP-modified CcO. 2' (or 3')-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP) binds tightly to CcO with a 1:1 stoichiometry (K_d ~ 3.5 μM, in 0.1 % Tween-80) and this binding is competitive with 8-azido-ATP modification of the enzyme (Lin and Chan, unpublished data). Accordingly, the extent of 8-azido-ATP labeling of a given CcO sample could be determined from comparison of the TNP-ATP binding between a sample of the enzyme that has

been subjected to irradiation in the presence of 8-azido-ATP and the native control by rapid filtration.

The affinity of native and 8-azido-ATP-modified CcO for TNP-ATP was determined by centrifuging a preequilibrated 2 ml solution of 30 μM of TNP-ATP with 10 μM of CcO (or 8-azido-ATP-modified CcO) in 5 mM Tris, 0.1 % Tween-80 buffer, pH 7.4, in a centricon-100 microconcentrator at 3,300 rpm for 10 min at 3°C. The microconcentrator retains CcO and its TNP-ATP complex but allows free TNP-ATP and detergent to pass the membrane. After the centrifugation, the concentration of TNP-ATP in the filtrate and the original solution together with that of CcO in the original solution were determined by Uv-Visible spectra.

Since the filtrate volume was about 20 % of the total volume, the species separated was assumed to reflect the concentration of the species in equilibrium in the original solution. Therefore, in these experiments on the binding of TNP-ATP to native or 8-azido-ATP-modified CcO, we have

$[A] = C_A^F$

$[E \cdot A] = C_A^O - C_A^F$

$[E] = C_E^O - [E \cdot A]$

in which [A] is the concentration of free TNP-ATP, [E] is the concentration of uncomplexed enzyme (CcO or 8-azido-ATP-modified CcO) and [E·S] is the concentration of the enzyme-TNP-ATP complex. C_A^F and C_A^O is the concentration of TNP-ATP in the filtrate and original solution respectively, C_E^O is the concentration of enzyme in the original solution.

Determination of the binding constant of cytochrome c to native CcO and 8-azido-ATP-modified CcO by rapid filtration method. To determine the binding constant of the cytochrome c to native and 8-azido-ATP-modified CcO, a preequilibrated solution of 10 μM of cytochrome c with 10 μM of native or 8-azido-ATP-modified CcO in 5 mM Tris buffer, pH 7.4 (low salt condition) or 5 mM Tris buffer with 100 mM KCl, pH 7.4 (high salt condition) was centrifuged in a centricon-100 at

3,300 rpm for 10 min at 3°C. The microconcentrator retains CcO but allows free cytochrome c to pass the membrane. After the centrifugation, the concentration of cytochrome c in the filtrate and the original solution, together with that of CcO in the original solution, were determined by UV-Visible spectrum. These data were analyzed in the same manner as in the above experiments on the complexation of TNP-ATP to native CcO or 8-azido-ATP-modified CcO.

Finally, to determine the effects of ATP and TNP-ATP on the binding constant between cytochrome c with native CcO, 10 mM ATP or 30 μM of TNP-ATP was also present in some of the low salt and high salt experiments with native CcO.

Steady-state kinetics of native and 8-azido-ATP-modified CcO. The steady-state turnover of CcO was determined by the polarographic method. 0.1 to 16 μM of cytochrome c was added to 5 nM of native or 8-azido-ATP-modified oxidase in 25 mM Tris, 5 mM ascorbate, 0.1% lauryl-D-maltoside, 0.7 mM TMPD, pH 7.4, and the turnover rate was monitored as the oxygen consumption rate with a VSI Model 53 Oxygen Electrode (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio). In one experiment with native CcO, free ATP was also added to a final concentration of 3 mM. The data are presented as Eadie-Hofstee plots. The activity is expressed in terms of the molecular turnover number (TN), where TN = mole cytochrome c/second per mole CcO at 20°C.

Transient electron transfer from cytochrome c to native CcO and 8-azido-ATP-modified CcO. Transient absorbance data were taken with an excimer dye laser at the Laser Facility of the Beckman Institute, California Institute of Technology. Electron transfer was initiated by the laser flash photolysis technique developed by Hazzard *et al.* (1991). When 5-deazariboflavin (5-DRF) is excited by a laser flash, it abstracts an electron from EDTA and becomes a flavin semiquinone. The flavin semiquinone rapidly reduces cytochrome c, which subsequently reduces CcO at a slower rate. The reoxidation of reduced cytochrome c by the oxidase was followed by monitoring the 550

nm absorption peak of the cytochrome c. The electron transfer rate was also followed by monitoring the reduction of cytochrome a of CcO at 604 nm. These experiments were performed on a solution containing 10 μM cytochrome c and 5-25 μM of CcO or 8-azido-ATP-modified CcO in a buffer of 5 mM Tris containing 1 mM EDTA, 0.1% lauryl maltoside, 33 μM 5-DRF, and 100 mM KCl, pH 7.4. In some experiments, ATP was also added to the buffer to a final concentration of 3 or 6 mM. The sample cuvette was sealed and degassed before being subjected to the excimer dye laser flash (BPBD at 395 nm). The optical signals represented the accumulation of 30 flashes.

Redox potentials of CcO and its 8-azido-ATP adduct. The effect of 8-azido-ATP modification on the redox potential of cytochrome a in CcO was measured by monitoring the following redox equilibrium for both native CcO and its 8-azido-ATP adduct:

Ferricyanide + Cytochrome a^{2+} <==> Ferrocyanide + Cytochrome a^{3+}

Solutions of $K_4(Fe(CN)_6).3H_2O$ (422mg) and $K_3(Fe(CN)_6)$ (329mg) were prepared in 10 mL each of degassed water. CcO (or 8-azido-ATP-modified CcO) was diluted with a 5 mM Tris, 100 mM NaCl, 0.1 % Lauryl-D-maltoside, pH 7.0 to a final concentration of 30 μM, total volume of 0.9 ml in a plastic cuvette. In some samples, ATP was added to the buffer to a final concentration of 3 mM. The samples then were sealed with rubber stopper and degassed with nitrogen gas for 10 min. A mixture of quinones ([1,3-Benzoquinone]: [1,2-Naphthoquinone]: [2-methyl^{-1},4-Naphthoquinone]: [2-hydroxy^{-1},4-Naphthoquinone] = 1:1:1:1, final concentration of 20 μM; Li *et al.*, 1992) was employed as redox-mediators in the redox titration. To initiate the redox titration, 100 μL of ferrocyanide stock solution was added to each of the samples and the visible spectra of the mixtures were recorded. Subsequently, ferricyanide was titrated into each of the samples in 2.5 μL increments with the visible spectrum recorded after each addition (a total of 9 data points were obtained). Following the titration, the fully reduced spectrum of CcO was taken by adding sodium

dithionide (5 mg in 100 µl) to the samples. The redox potential of cytochrome a was calculated from the following expression:

$$E^0_{\text{cytochrome a}} = 0.43 - 0.059 \log([\text{ferricytochrome a}]/[\text{ferrocytochrome a}] \times [\text{ferrocyanide}]/[\text{ferricyanide}]).$$

where we have taken the standard reduction potential for the ferricyanide/ferrocyanide couple to be 430 mV.

The redox potential of Cu_A was determined from the redox equilibrium:

$$Cu_A^{2+} + \text{ferrocytochrome c} \Longleftrightarrow Cu_A^{1+} + \text{ferricytochrome c}$$

First, the CcO (with 20 µM of redox mediators described above) was fully reduced by sodium dithionide. 20 µM of cytochrome c was then added to each of the samples. The above redox equilibrium was monitored as various amounts of ferricyanide was titrated into the solution. The redox potential of Cu_A was determined from the expression:

$$E^0_{Cu_A} = 0.265 - 0.0059 \log([Cu_A^{+1}]/[Cu_A^{+2}] \times [\text{ferrocytochrome c}] / [\text{ferricytochrome c}]).$$

The concentration ratios were determined from the Cu_A^{2+} and cytochrome c absorbances at 830 nm and 550 nm, respectively. The standard reduction potential of cytochrome c was taken to be 265 mV.

EPR Spectroscopy. EPR spectra were recorded on a Varian E-line Century series X-band spectrometer equipped with a 12 bit analog to digital converter used for the computer digitization of the signal. Sample temperature was maintained at 7 K by a liquid helium cryostat (Oxford

Instruments). Oxygen was removed from EPR samples by a single equilibration with argon gas immediately prior to freezing the sample.

RESULTS:

The extent of 8-azido-ATP modification of CcO. TNP-ATP forms a relatively strong 1:1 complex with CcO (K_d~3-4 μM in Tween-80) and this binding is competitive with 8-azido-ATP modification (Lin, J. and Chan, S. I., unpublished data). Modification of CcO with 8-azido-ATP reduces the apparent stoichiometry of binding of TNP-ATP to CcO (Table 1). The observed TNP-ATP binding stoichiometry suggests that 65 % of the oxidase molecules were labelled by 8-azido-ATP.

The effect of buffer ATP, TNP-ATP and 8-azido-ATP modification on the binding affinity of cytochrome c to CcO. It is well known that cytochrome c and CcO form a strong complex at low ionic strengths (K_d ~0.005 μM) and this interaction is strongly ionic strength dependent. As shown in Table 2, this interaction is also dramatically reduced (in excess of tenfold) upon 8-azido-ATP modification of the enzyme under low ionic strengths. However, the ionic strength dependence noted for the native enzyme becomes greatly suppressed. As expected, the association of cytochrome c to the modified oxidase is only sightly weaker than to native CcO under physiological ionic strengths.

A similar study of the effect of free (buffer) ATP on the interaction between the redox protein partners is not feasible in buffer, since the dissociation constant of ATP from CcO is only of the order of 2 mM (Bisson *et al.*, 1987; Lin and Chan, unpublished data) and it would take ATP concentrations in the 10 mM range to saturate the binding of ATP to CcO. Under these conditions, the ionic strength of the solution would approach 50 mM so that the interaction between cytochrome c and CcO is no longer in the low ionic strength regime. To complicate matters further,

Table 1. Apparent binding stoichiometry of TNP-ATP to native and 8-azido-ATP modified CcO at ionic strength of 5 mM

Sample	Apparent binding Stoichiometry ([TNP-ATP]:[CcO])
native CcO	0.95
8-azido-ATP modified CcO	0.36

Table 2. Binding of cytochrome c with native, and 8-azido ATP modified CcO at low[a] and high[b] ionic strength

Sample	Ka (at low salt)[a] (μM^{-1})	Ka (at high salt)[b] (μM^{-1})
native CcO	134.5	0.186
8-azido-ATP modified CcO	9.34	0.107
native CcO with 10 mM of free ATP		0.112
native CcO with 30 μM of TNP-ATP	8.45	0.139

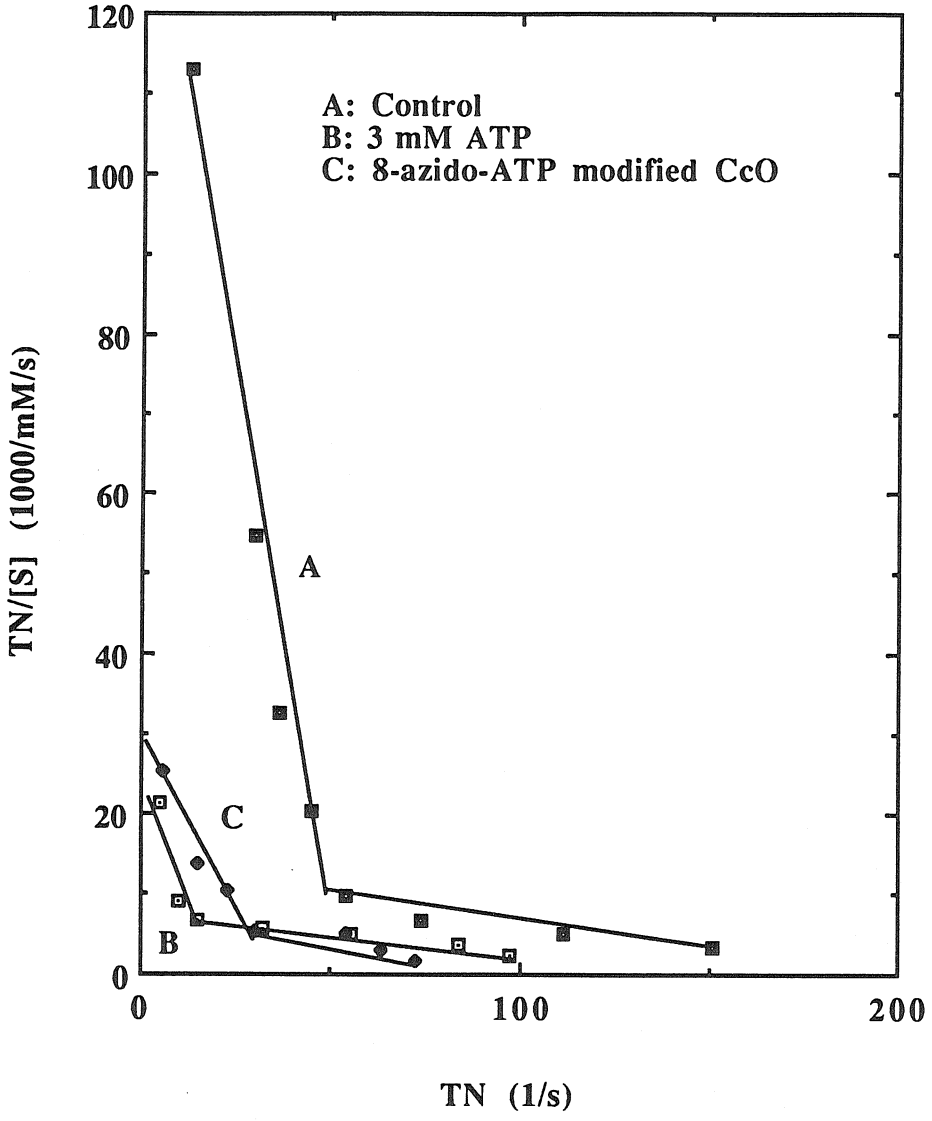
[a] ionic strength is 5 mM
[b] ionic strength is 105 mM

ATP binds directly to cytochrome c, also at mM concentrations (K_d ~ 5 mM), and this interaction is now known to interfere with the docking of cytochrome c with CcO. In the mitochondrion, these complexities do not obtain since ATP binds to CcO from the matrix side only. The cytochrome c-CcO interaction should be primarily sensitive to cytosolic ATP levels.

TNP-ATP binds to CcO, competing for the same site as 8-azido-ATP modification. Unlike ATP, however, its binding constant is much higher (in lauryl-D-maltoside, K_d = 1.6 μM; Reimann and Kadenbach, 1992; K_d = 0.46 μM; Lin and Chan, unpublished data). Accordingly, TNP-ATP could be exploited to examine the effect of ATP on the docking of cytochrome c to CcO under both low and high ionic strengths. As depicted in Table 2, TNP-ATP binding exhibits similar effects as 8-azido-ATP modification on the association of cytochrome c to CcO at low ionic strength: the interaction is about tenfold weaker compared to native CcO in both cases. Toward physiological ionic strengths, there is less disparity in the interaction between cytochrome c and the various CcO's, though the association of cytochrome c with CcO is still stronger with native CcO than in the presence of ATP, TNP-ATP, or upon 8-azido-ATP modification.

Steady-state turnover of CcO and its 8-azido-ATP adduct. The steady-state kinetics of native and its 8-azido-ATP adduct are represented as Eadie-Hofstee plot in Figure 1. For comparison, the results for native CcO in the presence of 3 mM ATP are also included. At this concentration of ATP, about 75 % of the oxidase molecules have one ATP bound. Since about 65 % of the oxidase molecules in the 8-azido-ATP-modified CcO preparation are labelled, the steady-state kinetics observed for this preparation should be directly comparable to that for the CcO preparation in the presence of 3 mM ATP. Indeed, the two samples exhibit very similar Eadie-Hofstee plots, indicating that 8-azido-ATP modification of CcO has a similar inhibitory effect on CcO as non-covalent ATP binding. For both of these preparations, the activity of the high-affinity phase was inhibited, and the activity of the low-affinity phase was also reduced. These results are consistent

Figure V.1. The effects of 8-azido-ATP modification and ATP binding on the steady-state kinetics of CcO. The activity of native CcO, native CcO in the presence of 3 mM ATP, and 8-azido-ATP-modified CcO was assayed as detailed in the Materials and Methods section. (A) native CcO (control); (B) CcO in the presence of 3 mM ATP; (C) 8-azido-ATP-modified CcO.


A: Control
B: 3 mM ATP
C: 8-azido-ATP modified CcO
A
B
C
120
100
80
60
40
20
0
0
100
200
TN/[S] (1000/mM/s)
TN (1/s)

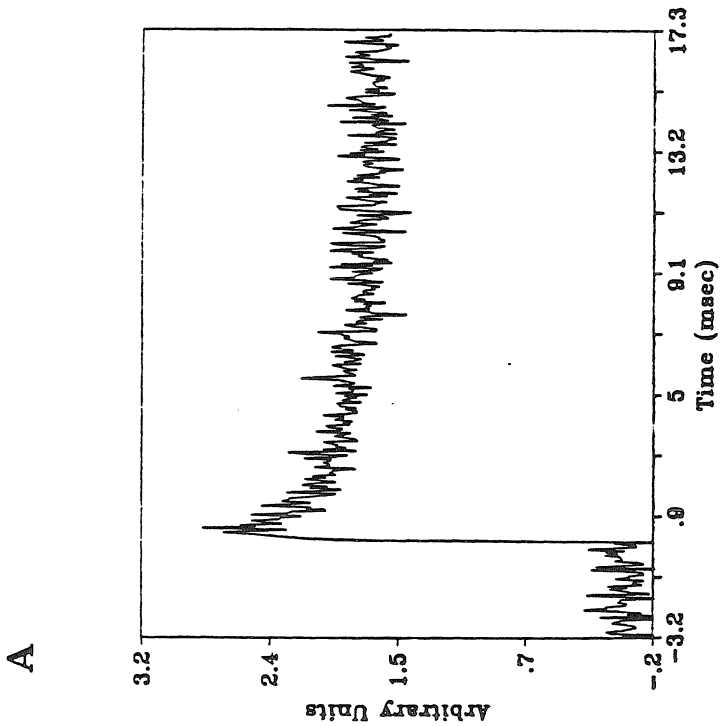
with the earlier experimental observations of Ferguson-Miller *et al.* (1976) and Bisson *et al.* (1987).

Transient absorption study of the electron input from ferrocytochrome c to CcO under various conditions. Figure 2A show typical transient kinetic traces observed for the intracomplex electron transfer between horse cytochrome c and fully oxidized native bovine CcO at 1:1 mole ratio and 105 mM ionic strength. The reduction of ferricytochrome c by 5-DRF semiquinone and its subsequent reoxidation by CcO were monitored at 550 nm (Figure 2A). The kinetic trace corresponding to the cytochrome c reoxidation is biphasic and fits well to a sum of two exponentials, as noted earlier (Pan, *et al.*, 1991). An observed kinetic constant of 872 s^{-1} is obtained for the fast phase. The reduction of cytochrome a in the CcO was followed at 604 nm (Figure 2A). A kinetic constant of 895 s^{-1} was obtained for the fast phase here.

Under otherwise identical conditions, the 8-azido-ATP-modified CcO display somewhat slower electron transfer kinetics. Figure 2B shows the reoxidation of cytochrome c and the reduction of cytochrome a for the 8-azido-ATP-modified CcO. An apparent kinetic constant of 518 s^{-1} was observed for the fast phase of the reoxidation of the cytochrome c and an apparent kinetic constant of 542 s^{-1} was observed for the reduction of cytochrome a. However, since the 8-azido-ATP-modified CcO preparation includes 35 % unlabelled oxidases, the actual electron input rates must be slower than the values indicated by the apparent rates. Similar data are obtained for native CcO in the presence of 3 mM and 6 mM ATP, the apparent rate constants for the reoxidation of cytochrome c are 412 s^{-1} and 362 s^{-1}, respectively, and the corresponding rate constants for the reduction of cytochrome a are 426 s^{-1} and 366 s^{-1}.

As controls, a Cu_A-depleted CcO was also investigated. The kinetic constants deduced form the transients at 550 nm and 604 nm for a sample for which the Cc/CcO mole ratio is 1:1 are 335 s^{-1} and 347 s^{-1}, respectively (data no shown). A sample of the 8-azido-ADP-modified CcO was also

Figure V.2. Intracomplex electron transfer from ferrocytochrome c to native CcO, the CcO-ATP complex, and 8-azido-ATP-modified CcO. Left panels: Reduction of cytochrome c by photogenerated flavin semiquinone and reoxidation of ferrocytochrome c by various CcO's followed at 550 nm. Right panels: Reduction of cytochrome a in the various CcO's by the ferrocytochrome c observed at 604 nm. (A) native CcO; (B) native CcO in the presence of 3 mM ATP; (C) 8-azido-ATP-modified CcO. Experimental conditions: Concentration of various CcO: 10 μM; cytochrome c concentration: 10 μM; ionic strength: 105 mM. Other experimental conditions are detailed in the Materials and Methods section.


A
Arbitrary Units
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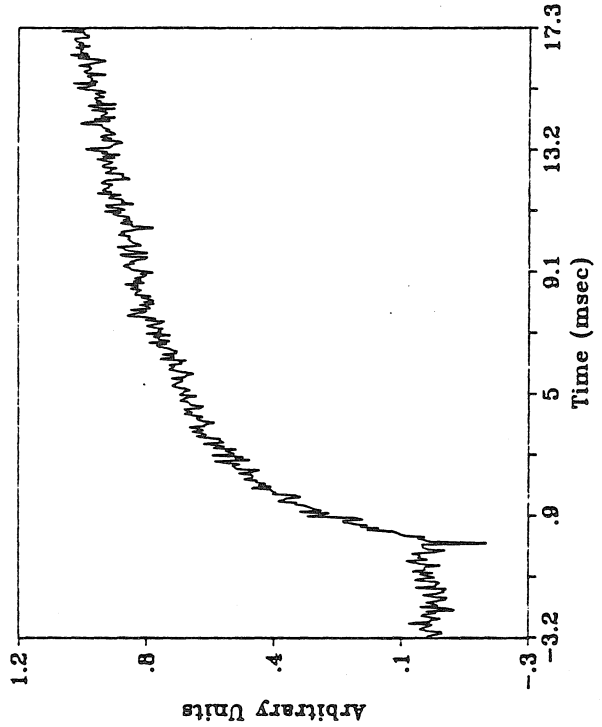
1.5
.7
-.2
-3.2
.9
5
9.1
13.2
17.3
Time (msec)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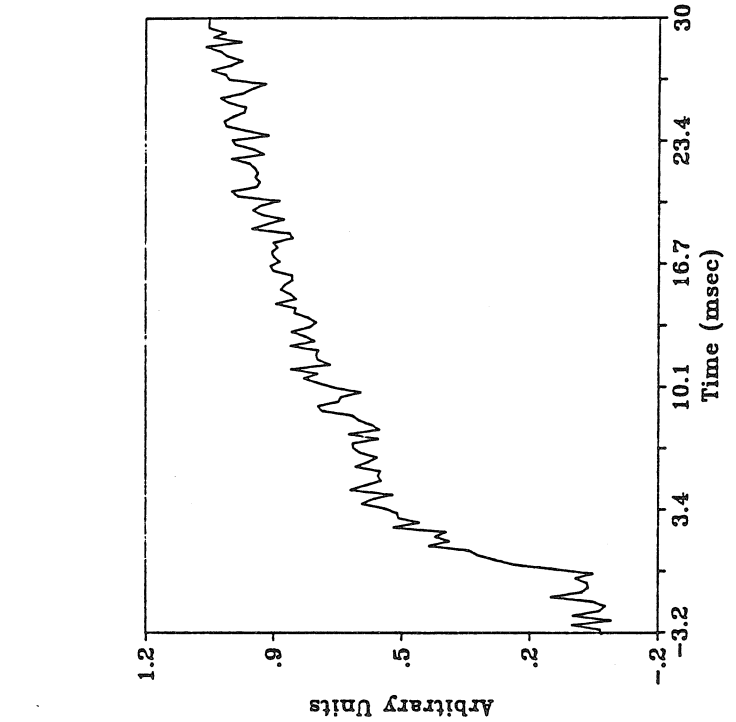
17.3
Time (msec)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.6
.2
-.2
Arbitrary Units
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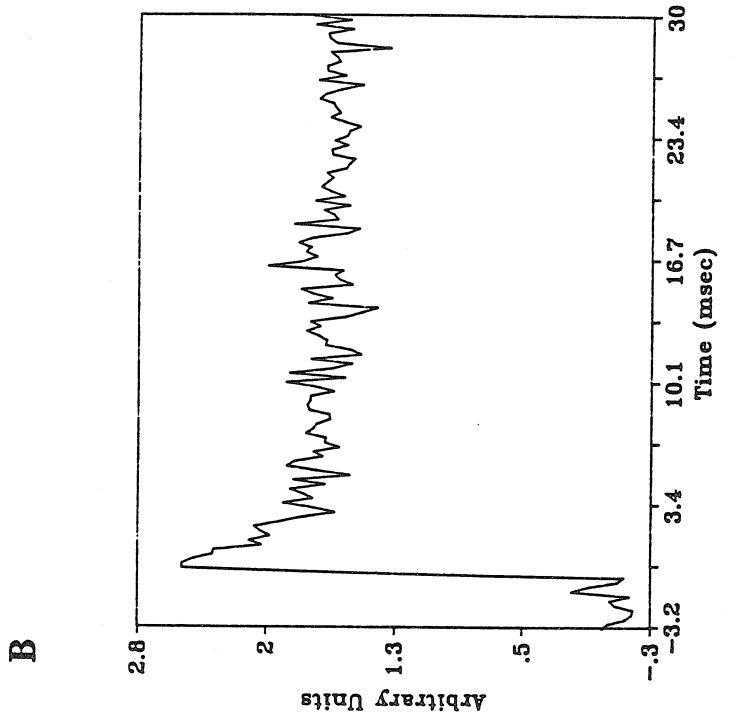
3.4
10.1
16.7
23.4
30
Time (msec)

B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Arbitrary Units
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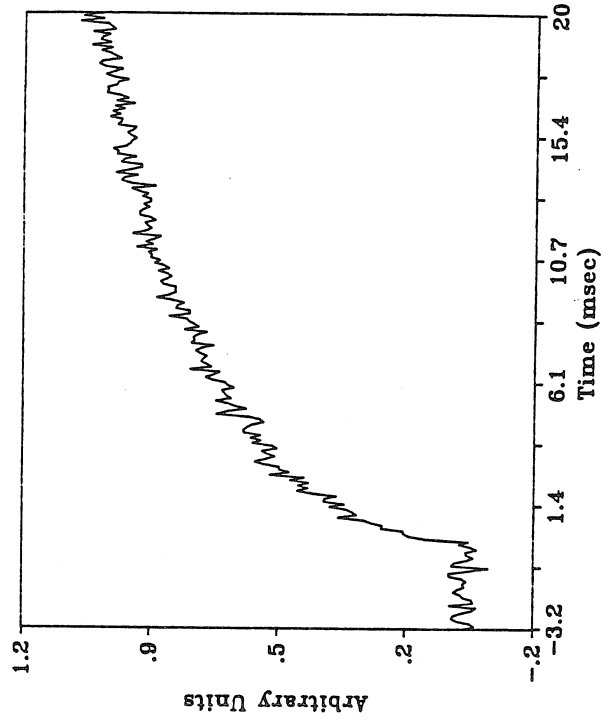
3.4
10.1
16.7
23.4
30
Time (msec)


1.2
.9
.5
.2
-.2
Arbitrary Units
-3.2
1.4
6.1
10.7
15.4
20
Time (msec)

C

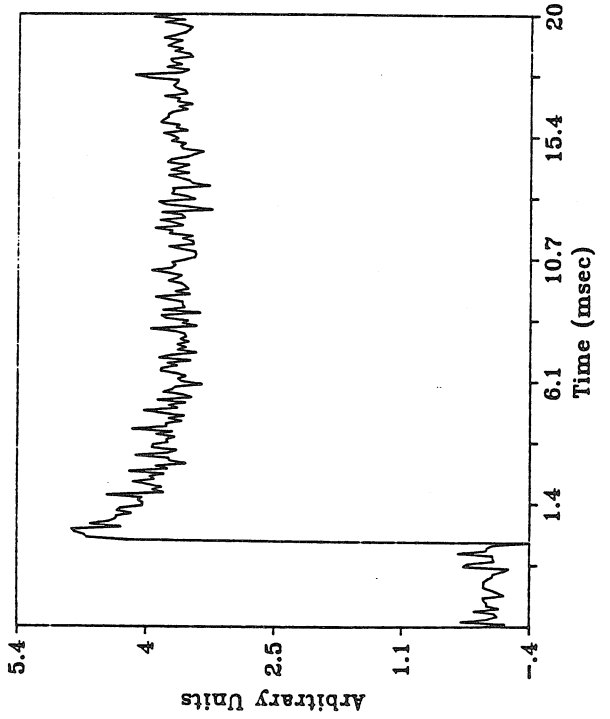

5.4
4
2.5
1.1
-.4
Arbitrary Units
1.4
6.1
10.7
15.4
20
Time (msec)

Table 3. observed rate constant for electron transfer from cytochrome c to heme a of CcO ([Cc]:[CcO]=1:1)

Sample	k_{obs} (s^{-1})	k_{obs} /$k_{obs(native)}$ (%)
native CcO	883	100
8-azido ATP modified CcO	530	60
8-azido ADP modified CcO	796	90
native CcO in the presence of 3mM ATP	419	47
native CcO in the presence of 6mM ATP	364	41
Cu_A depleted CcO	341	39

Cc cytochrome c

studied. Here, we obtain an apparent rate constant of 796 s^{-1} under otherwise identical conditions. These data are also included in Table 3.

The above transient kinetic experiments for native CcO, 8-azido-ATP-modified CcO, and CcO in the presence of 3 mM ATP have been repeated for various oxidase concentrations under otherwise identical conditions including the ionic strength. These data are summarized in Figure 3. From the kinetic constants observed at different oxidase concentrations, we have estimated the apparent association constant for formation of the ferrocytochrome c: CcO complex and the first-order rate constant for the intracomplex electron transfer (Pan *et al.*, 1991) for each of the three enzyme preparations. The equation for fitting the kinetic data is

$$k_{obs} = k_{et} K_a [CcO]_{ox} / (K_a[CcO]_{ox} + 1) \quad (1)$$

where K_a is the apparent association constant for the formation of the ferrocytochrome c:CcO complex and k_{et} is the intracomplex electron transfer rate constant. When the concentration of CcO is sufficiently high,

$$k_{obs} = k_{et} \quad (2)$$

so that the maximal observed rate approximates the intracomplex electron transfer rate.

The best fits of the kinetic data obtained for native, 8-azido-ATP-modified CcO, and CcO in the presence of 3 mM of ATP are shown in Figure 3 and the results are tabulated in Table 4. We see that both ATP binding or covalent attachment of ATP to the ATP-binding site of CcO decrease the affinity of CcO for cytochrome c. K_a is reduced by 35 % in the case of 8-azido-ATP-modified CcO and 53 % for CcO with 3 mM ATP present. The intracomplex electron transfer rate constant is also influenced by ATP binding and 8-azido-ATP modification. Compared to native CcO, k_{et} has been reduced to 69 % and 72 % for the 8-azido-ATP-modified and ATP-bound enzymes, respectively. It should be noted that these numbers represent upper limits since the binding of ATP to the oxidase is not fully saturated at 3 mM ATP and the 8-azido-ATP-modified preparation is no better than 70 % labelled. In addition, it should be noted that k_{et} for the native CcO (1430 s^{-1}) obtained in

Figure V.3. Kinetics of electron transfer between ferrocytochrome c (10 μM) and various CcO's as a function of CcO concentration (5-25 μM) at an ionic strength of 110 mM. The reaction conditions are described in the Materials and Method section. 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 CcO. The solid curves represent the best fits of the data to Equation (3). (A) native CcO; (B) CcO in the presence of 3 mM ATP; and (C) 8-azido-ATP-modified CcO.

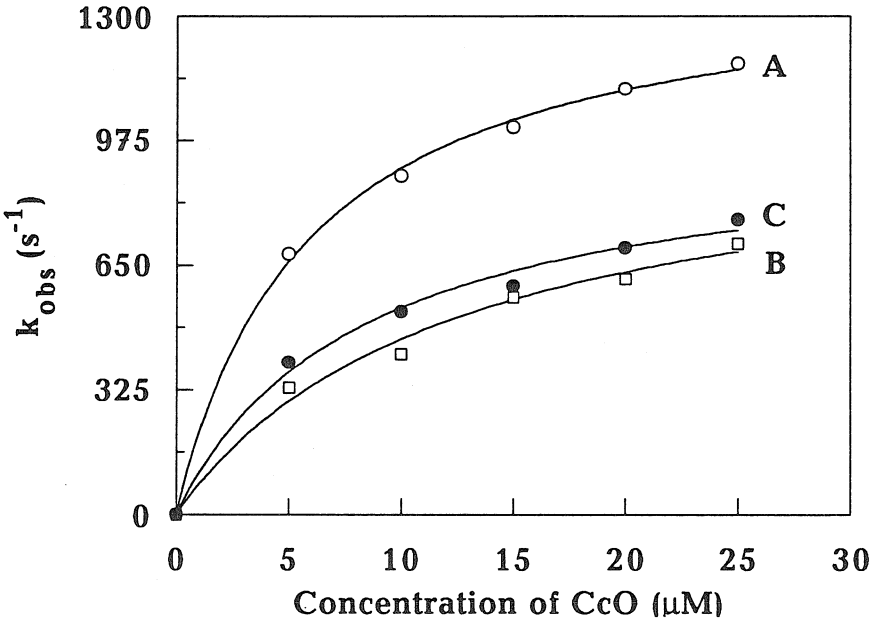

1300
975
650
325
0
k_{obs} (s^{-1})
0
5
10
15
20
25
30
Concentration of CcO (μM)
A
C
B

Table 4. kinetic parameters for intracomplex electron transfer between cytochrome c and cytochrome c oxidase

Sample	Ka [x 10^{-4} (M^{-1})]	k_{et} (s^{-1})
native CcO	3.4	1430
8-azido ATP modified CcO	2.2 (1.5)	990 (712)
native CcO in the presence of 3 mM ATP	1.6 (0.8)	1030 (733)

this study is lower than that observed in our earlier study (2580 s^{-1}) (Pan *et al.* 1991). Since, the intracomplex electron transfer rate constant does vary from batch to batch of enzyme, it could be that the activity of the oxidase in the batch used in the present study is lower. Our present value of k_{et} is close to that previously reported by Hazzard *et al.* (1470 s^{-1}) (1991).

In an effort to obtain more reliable measures of the electron input rates for the modified and ATP-bound CcO, we have corrected for the contribution of the unlabelled or uncomplexed enzyme to the observed transients. Based on the existence of two CcO species and assuming that the on-off rates of the cytochrome c is rapid compared to the intracomplex electron transfer rates for the two possible types of complexes, the observed rate constant should be given by

$$k_{obs} = k_{et} K_a [CcO]_{ox} F_1 / (K_a[CcO]_{ox}F_1 (1+(K_a'[CcO]_{ox}F_2(1+k_{et}/k_{-1})) /(K_a[CcO]_{ox}F_1 (1+k_{et}'/k_{-1}')))+ 1) + k_{et}' K_a' [CcO]_{ox}F_2 / (K_a'[CcO]_{ox}F_2(1+(K_a[CcO]_{ox}F_1(1+k_{et}'/k_{-1}')) /(K_a'[CcO]_{ox}F_2 (1+k_{et}/k_{-1})))+ 1) \quad (3)$$

where K_a and K_a' are the formation constants for cytochrome c complexes between native CcO and 8-azido-ATP-modified (or ATP-bound) CcO, respectively; k_{et} and k_{et}' denote the intracomplex electron transfer rates within the native and modified (or ATP-bound) CcO's; k_{-1} and k_{-1}' denote the off-rates of the cytochrome c from native and modified (or ATP-bound) CcO's; and F_1 and F_2 are the percentage of unmodified (unbound) and modified (ATP-bound) CcO in the samples.

Using the k_{et} and K_a deduced previously for the native enzyme for the value of k_{et} and K_a in equation 3, the data for 8-azido-ATP-modified CcO and native CcO in the presence of 3 mM ATP could be corrected for the unmodified and uncomplexed enzyme. Since $K_a \sim K_a'$ under high ionic strengths, we can assume that (1) either $k_1 \sim k_1'$ or (2) $k_{-1} \sim k_{-1}'$ without introducing significant error. The result of the best fits of the kinetic data obtained for 8-azido-ATP-modified CcO and CcO in the presence of 3 mM of ATP, assuming 65 % of the CcO is modified in the 8-azido-ATP-modified CcO and 75 % of the CcO is bound in the native CcO in the presence of 3 mM ATP, are also tabulated in parenthesis in Table 4. After the correction, K_a is reduced by 56 % in the case of

8-azido-ATP-modified CcO and 76 % for CcO with 3 mM ATP present. The intracomplex electron transfer rate constant is also influenced by ATP binding and 8-azido-ATP modification. Compared to native CcO, k_{et} is reduced by 50 % and 51 % for the 8-azido-ATP-modified and ATP-bound enzyme, respectively.

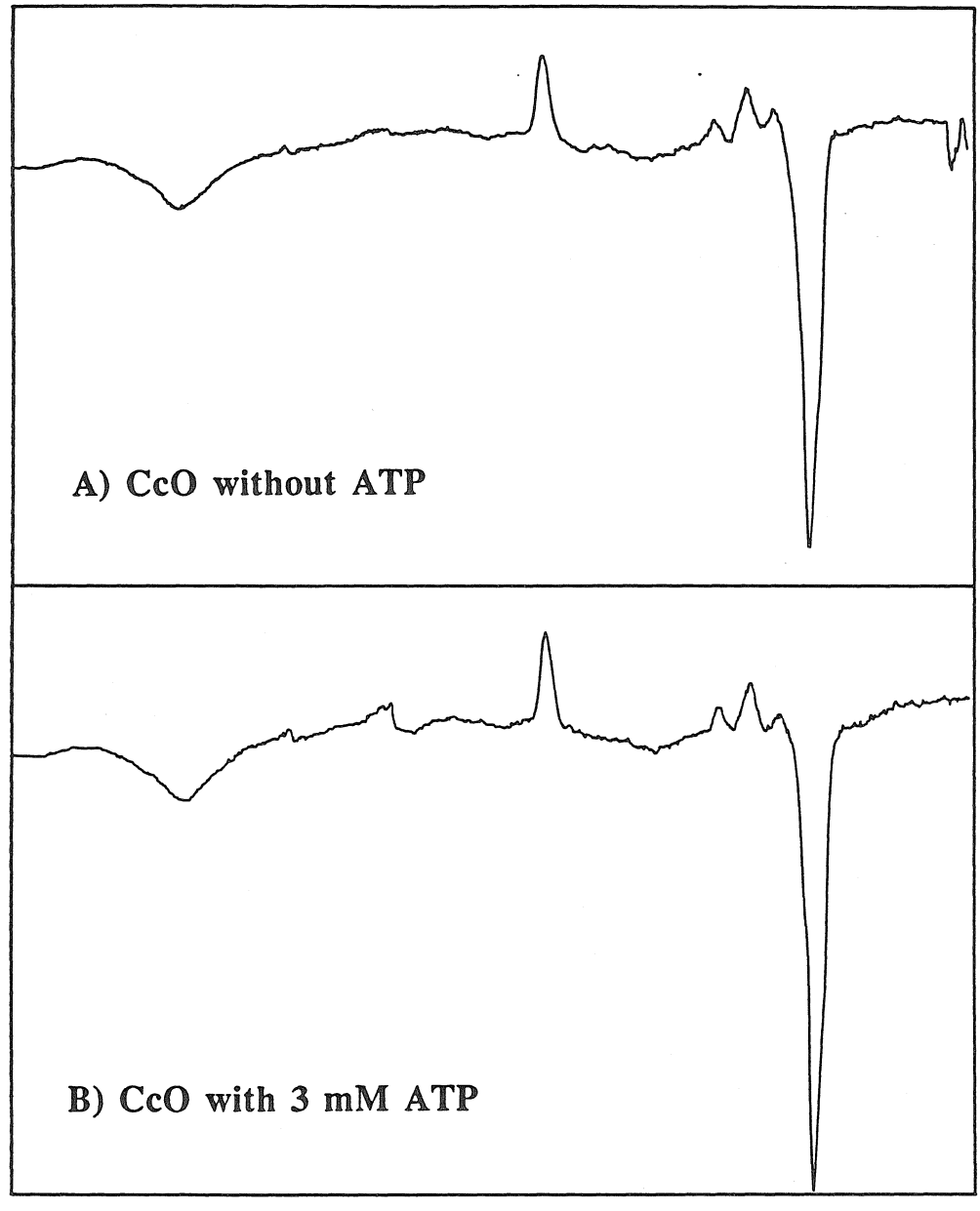
The effects of ATP-binding and 8-azido-ATP modification on the redox potentials of the low potential centers in CcO. In order to ascertain the origin of the slower k_{et} observed for CcO in the presence of 3 mM ATP or for 8-azido-ATP-modified CcO, we have measured the redox potentials of cytochrome a and Cu_A under these conditions. For comparison, the redox potentials in the presence of 3 mM of ADP have also been determined. These results are summarized in Table 5. No significant changes in the redox potentials were uncovered for either cytochrome a or Cu_A. It should be noted that these redox potentials for cytochrome a and Cu_A have been obtained for quite distinct states of oxidase. The electrons are titrated into the fully oxidized enzyme in the experiments on cytochrome a, whereas they are being removed from Cu_A in the experiments involving Cu_A. Accordingly, a direct comparison between the two sets of potentials may not be meaningful due to redox interactions among the metal centers within the oxidase. In any case, these results indicate that the slower k_{et} observed upon 8-azido-ATP modification or ATP binding could not be due to a change in the redox potentials of cytochrome a or Cu_A.

EPR Spectra of CcO in the presence of ATP. We have recorded the EPR spectra of native CcO in the presence of 3 mM ATP at 7 K (Figure V.4) . These EPR signals for Cu_A and cytochrome a are identical to those for the enzyme in the absence of ATP. Thus, ATP-binding, and by inference, 8-azido-ATP modification also, do not perturb the ligand environments of cytochrome a or Cu_A significantly. These spectroscopic conclusions augment those derived earlier from the redox potential measurements. Taken together, we must conclude that the change in k_{et} upon ATP binding or 8-azido-ATP modification arises from changes in the docking of the cytochrome c to the oxidase.

Table 5. redox-potentials of heme a and Cu_A of CcO

Sample	Redox-potential of Heme a (mV)	Redox-potential of Cu_A (mV)
native CcO	321	264
8-azido-ATP-modified CcO	325	275
native CcO in the presence of 3mM ATP	322	252
native CcO in the presence of 3mM ADP	317	278

Figure V.4. EPR spectra of Cu_A and heme a for CcO (A) without ATP , (B) with 3 mM ATP. Sample temperature, 7 K; microwave frequency, 9.16 GHz; microwave power 5 mW; modulation amplitude, 10 G


A) CcO without ATP
B) CcO with 3 mM ATP
0
4000
Magnatic Field (Gauss)

DISCUSSION:

We have shown in this study that 8-azido-ATP modification of CcO influences dramatically the electron input from ferrocytochrome c to the oxidase. To illustrate that the chemical modification of the oxidase mimics the direct binding of ATP, we have compared the effects of 8-azido-ATP modification with the effects of ATP binding in the presence of 3 mM and 6 mM ATP. The effects of ATP binding and 8-azido-ATP modification on the electron input as well as the steady-state activity of the enzyme were found to be essentially identical. In contrast, neither the steady-state activity of CcO nor the electron input from ferrocytochrome c are significantly affected by ADP binding (in the presence of 3 mM ADP) or 8-azido-ADP modification.

The use of 8-azido-ATP modification obviates any non-specific ionic strength effects or any direct interaction between ATP and cytochrome c on the docking of cytochrome c to CcO. Wallace and coworkers (Craig and Wallace, 1991, 1993) have noted that ATP binds directly to cytochrome c under sufficiently high ATP concentrations and have suggested that this ATP-cytochrome c interaction has deleterious effects on the interaction between cytochrome c and CcO. However, since the K_d for the binding of ATP to cytochrome c has been estimated to be of the order of 3.3 and 4 mM for oxidized and reduced cytochrome c, respectively (Craig and Wallace, 1991), any effects arising from this specific cytochrome c-ATP interaction should be fairly minimal at the concentration of ATP (3 mM) employed in the present studies. As a further control, we have examined the effect of TNP-ATP binding on the docking of cytochrome c to CcO. Since K_d for the CcO-TNP-ATP complex is of the order of 1 μM and the corresponding K_d for the cytochrome c-TNP-ATP complex is only slightly lower (~80%) than that with ATP (Lin and Chan, unpublished data), any effects observed here for TNP-ATP should be specific to CcO. Observations identical to those noted for 8-azido-ATP modification or ATP-binding (3 mM ATP) have been obtained for

TNP-ATP. Thus, the effects being reported here are specific to ATP binding to its binding site in CcO.

8-azido-ATP modification of (as well as ATP and TNP-ATP binding to) CcO decrease the binding affinity of CcO for cytochrome c and slows down the electron transfer ferrocytochrome c to CcO. The effects are particularly striking at low ionic strengths. Under these conditions, the binding affinity of cytochrome c to CcO is reduced by more than a factor of 10. Even under physiological ionic strengths, transient absorption measurements indicate a decrease in the cytochrome c binding constant (about a factor of 2) as well as the intracomplex electron transfer rate constant. These results are consistent with the effects of ATP binding or 8-azido-ATP modification on the steady-state activity of the oxidase. Both ATP binding and the 8-azido-ATP modification of the enzyme exhibit similar inhibition of the high-affinity phase of the Eadie-Hofstee kinetic plots; in addition, V_{max} of the low-affinity phase is reduced. The inescapable conclusion that one could draw from all these data, taken together, is that the docking interaction between cytochrome c and CcO has been altered upon 8-azido-ATP modification of or ATP binding to the oxidase. The strikingly different behavior of the cytochrome c:CcO complex toward ionic strength between the native oxidase and the ATP-bound or 8-azido-ATP-modified oxidase provides unequivocal evidence in support of the change in the docking interaction postulated here.

The electron input from ferrocytochrome c to CcO is highly complex. Aside from the details of the docking of the cytochrome c to the oxidase, which could influence the electron transfer pathway(s) and the rate(s) of the electron transfer, the problem is further complicated by the possibility of multiple electron input ports. Recent experiments indicate that Cu_A is the primary electron acceptor from ferrocytochrome c; however, the electron could also be transferred directly into cytochrome a (Pan *et al.*, 1991). It appears that both the rates as well as the branching ratio of the electron input are sensitive to the docking. Thus, under low ionic strengths, when the tight Cc:CcO complex is formed, the electron input is primarily to Cu_A and this input is extremely facile with rates

approaching 10^5 s^{-1} (Pan *et al.*, 1993). In the case of the first electron, this electron is then subsequently transferred to cytochrome a with a rate of the order of 15,000 s^{-1} (Morgan *et al.*, 1987; Kobayashi *et al.*, 1987; Pan *et al.*, 1993). Under more physiological ionic strengths, however, the electron input rate is slower (1,000-3,000 s^{-1}) (Hazzard *et al.*, 1991; Pan *et al.*, 1991; Larsen *et al.*, 1992; Pan *et al.*, 1993). In addition, the electron from ferrocytochrome c could also be transferred directly to cytochrome a (Pan *et al.*, 1991). If so, it is clear that the observed rates reflect both the intrinsic rates into the two electron acceptors, namely Cu_A and cytochrome a, as well as branching ratio of the electron input through the two ports.

We believe that the data that we have compiled here on the effects of ATP binding or 8-azido-ATP modification of CcO are to be interpreted in the above light. More specifically, we propose that ATP binding or 8-azido-ATP modification allosterically modifies the binding domain(s) on the oxidase for ferrocytochrome c, and that the observed electron input rates reflect changes in the branching of the electron input away from Cu_A toward cytochrome a as a consequence of the altered docking of the cytochrome c to the oxidase. Bisson *et al.* (1987) have previously noted a change in the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide metho-*p*-toluenesulfonate (CMC) modification efficiency of the carboxyl ligands of subunit II in the presence of ATP and have concluded that ATP binding to CcO causes an allosteric conformational change in the vicinity of the cytochrome c binding site, presumably subunit II. The observed electron input rates for the CcO-ATP complex and the 8-azido-ATP-modified enzyme are consistent with the hypothesized change in the branching of the electron input branching from Cu_A toward cytochrome a. These cytochrome a electron transfer rates approach those for the Cu_A-depleted oxidase, where the electron can be transferred from ferrocytochrome c directly to cytochrome a only.

Electron transfer processes in biological systems are complicated, particularly those involving redox protein partners such as the system under consideration here. Aside from the redox

potentials of the electron donor and acceptor, the rates are strongly dependent on the electronic coupling between the redox partners, which can in turn be controlled by the distance (Moser *et al.*, 1992), as well as the nature of the medium between them (Wuttke *et al.*, 1992; Beratan *et al.*, 1991). In the present instance, the redox potentials of neither the donor nor the two possible electron acceptors are affected by ATP binding or 8-azido-ATP modification. Thus, the observed change in the electronic coupling between the electron donor and the two potential electron acceptors results from the change in the docking between the redox protein partners. Under physiological conditions, the problem is further complicated by the possibility of a distribution of docking conformations instead of one unique docking conformation in the complex at low ionic strength.

The results of the present study are significant and can have the following important implications. It is apparent that CcO is equipped with a chemical machinery that is capable of sensing the level of ATP (in the matrix) in the mitochondrion. Upon the binding of ATP, it appears that CcO can undergo a conformational transition to modulate the exposure of those domains of the protein that are important in the recognition of cytochrome c. Through such an allosteric mechanism, the oxidase can alter the docking of the cytochrome c and steer the shuttling of the electrons into the protein more toward cytochrome a. There are two outcomes of this allosteric control that should be underscored here. First, both the binding of the cytochrome c to the oxidase as well as the electron transfer rates are reduced so that the electron input rates become more closely matched to the turnover of the enzyme. Second, Cu_A is bypassed as the primary electron input port.

Although the two low potential centers appear to be in rapid redox equilibrium (Morgan *et al.*, 1987; Kobayashi *et al.*, 1987; Pan *et al.*, 1993), it appears that the enzyme is capable of tuning their relative redox potentials during turnover. As an example, the reduction potential of cytochrome a is as much as 120 mV more positive than that of Cu_A in the fully oxidized resting oxidase (Li *et al.*, 1991); however, when the binuclear site becomes one-electron reduced, this

reduction potential difference decreases to about 60 mV, with the potential of cytochrome a still more positive than that of Cu_A (He, Q.-Z., Pan, L.-P., and Chan, S. I., unpublished data). In addition, there is experimental evidence suggesting that the reduction potential of cytochrome a eventually drops below that of Cu_A at some point during the turnover cycle (Brzezinski and Malmstrom, 1986). Chan and Li (Chan and Li, 1990) have previously suggested that the intramolecular electron transfer from cytochrome a and Cu_A might be differentially linked to proton pumping. If so, the two electron transfer pathways may be exploited to regulate the efficiency of the free energy transduction, enabling the enzyme to adapt to varying energetic demands of the cell. It might be that, under high ATP levels, those electron transfers that are linked to proton pumping are suppressed, and electron leak pathways are populated instead to shuttle the electrons from the low potential metal centers to the dioxygen reduction site. It is well known that the H^+/e^- ratio is diminished at high ATP levels. The common interpretation of these data is that the redox linkage is accompanied by high proton slippage. However, another possible scenario is that the proton pumping machinery has been disengaged from electron transport. Indeed, for an electron driven proton pump that is also intimately coupled to ATP-synthesis, which CcO appears to be, it seems rather unlikely that the free energy transducer is not equipped with a negative feedback system with some of the very properties uncovered here. A redox-linked proton pump that responds to ATP levels by simple molecular slippage in our judgment lacks the robustness to meet the variety of energetic demands that a typical cell is normally subjected to.

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Chapter V

The Binding of TNP-ATP to Cytochrome c Oxidase-Detergent Micelles

ABSTRACT:

The interaction of ATP with bovine heart cytochrome c oxidase (CcO) has been studies using the fluorescence nucleotide analogs, 2, (or 3')-O-(2, 4, 6-trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP). TNP-ATP forms a relatively strong 1:1 complex with CcO (K_d = 4 μM); the corresponding ATP complex is about 1000-fold weaker (K_d = 2 mM). To investigate the origin of this disparity in the binding affinity between ATP and TNP-ATP, we have compared the partitioning of TNP-ATP, 2, (or 3')-O-(2, 4, 6- trinitrophenyl) adenosine 5'-monophosphate (TNP-AMP), and ATP into various detergents commonly employed to solubilize CcO (Tween-80, Triton X-100, Brij 35, and lauryl-D-maltoside), as well as their binding to various CcO-detergent micelles by the fluorescence titration and rapid filtration methods. Fluorescence titrations were also undertaken at different temperatures in order to obtain estimates of the thermodynamic parameters associated with the binding. Both the binding of TNP-ATP to detergent and to CcO-detergent micelles show similar detergent dependences, suggesting that interaction of the hydrophobic trinitrophenyl (TNP) moiety with the detergent contributes significantly to the free energy of binding of TNP-ATP to CcO-detergent micelles. On the basis of these results, we propose that TNP-ATP binds to CcO via the triphosphate linkage with the TNP fluorophore embedded in the detergent. This conclusion is supported by the different effects of TNP-ADP and TNP-AMP on the activity of CcO compared with TNP-ATP. The binding of TNP-ATP to CcO exhibits similar effects on the steady state kinetics between cytochrome c and CcO as 8-azido-ATP modification. Since the binding of ATP to CcO appears to involve subunit IV, the role of the putative magnesium ion that is associated with subunit in the binding will be discussed

INTRODUCTION:

Cytochrome c oxidase (CcO) is the terminal enzyme 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 mitochondria. As an intricate molecular machine, it has the capability to respond to various conditions of the mitochondrion, including protonmotive force, varying electron pressures, as well as cytosolic and matrix ATP levels. In fact, the manner in which various oxidases respond to matrix ATP is rather intriguing and this information could in principle shed some light on the electron transfer step(s) that are linked to proton pumping.

Ferguson-Miller *et al.* (1976) and Hess (1977) were the first to demonstrate that ATP can affect the activity of CcO. These workers showed that ATP, in millimolar concentrations, inhibit the electron transfer from ferrocytochrome c to dioxygen in bovine heart CcO (Ferguson-Miller *et al.*, 1976) and yeast CcO (Roberts and Hess, 1977), and proposed a regulatory role for ATP on the function of CcO in vivo. Montecucco *et al.* (1986) attempted to locate the ATP the ATP binding site by photocrosslinking CcO with the ATP analog, 8-azido-[γ-^{32}P]ATP, and observed labeling of subunit IV and VII, concluding that subunit IV and one of the subunit VII polypeptides are probably involved in the binding of ATP. On the other hand, Bisson *et al.* (1987) demonstrated that certain surface carboxyls of subunit II, that are important in the docking interactions between cytochrome c and CcO, become less accessible toward modification by the water soluble carbodiimides 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide metho-*p*-toluenesulfonate (CMC) in the presence of ATP. One possible interpretation of these data, taken together, is that the binding of ATP to CcO has led to an allosteric transition that has altered the surface exposure of some of the carboxyls on the cytochrome c binding domains of subunit II.

The effects of ATP on the activity of CcO are actually quite complex. Aside from the allosteric effects induced by ATP binding from the matrix side, cytosolic ATP also affects the Cc:CcO interactions via nonspecific ionic strength effects. In addition, Wallace and coworker (Craig and Wallace, 1991, 1993) have also indicated that ATP can bind directly to Cc and this interaction can have deleterious effects on the docking of Cc to CcO. Huther and Kadenbach (1986 and 1987) attempted to distinguish between the specific allosteric effect of ATP from nonspecific ionic strength effects by (1) using 8-azido-ATP covalently modified CcO for the kinetic studies, and (2) exploiting the use of reconstituted CcO membrane vesicles to sort out the effects arising from extraliposomal and intraliposomal ATP. The 8-azido-ATP-modified CcO showed similar kinetic effects as free ATP (Huther and Kadenbach, 1986). When the ATP effect is studied with reconstituted CcO vesicles, extraliposomal ATP increases Km for cytochrome c similar to intraliposomal ATP (Huther and Kadenbach, 1987); however, the two ATP interactions otherwise showed quite different characteristics. The interaction of ATP with CcO on the cytosolic side is nonspecific, non-saturable, and appears to be solely electrostatic; the ATP interaction from the matrix side is specific and saturable. Interestingly, a similar kinetic effect of ATP is observed for both *Paracossus dentrificans* and bovine heart CcO when the ATP is added to the cytosolic side of the reconstituted vesicles; in contrast, no influence of nucleotides was observed with the *Paracoccus* enzyme in the presence of matrix ATP (Huther and Kadenbach, 1988a). It is evident that the interaction of matrix nucleotides with CcO involves the nuclear encoded subunits only; these nuclear encoded subunits are lacking in CcO from *Paracossus dentrificans*. Stowell *et al.* (1993) have suggested, on the basis of comparative electron input studies between the bovine and *Paracossus* enzymes, that the mechanism of ATP feedback is in fact quite different between the two enzymes.

Another approach, championed also by the Kadenbach laboratory (Reimann and Kadenbach, 1992) in the study of the interaction of CcO with matrix ATP, is to substitute ATP by the fluorescent analog ' (or 3')-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP). TNP-

ATP has been found to be useful in earlier studies on the specific binding of ATP to heavy myosin ATPase (Hiratsuka and Uchida, 1973) and mitochondrial ATP synthase (Grubmeyer and Penefsky, 1981; Garboczi *et al.*, 1988a, b) because of its characteristic absorption and fluorescence properties upon association with a hydrophobic environment. Reimann and Kadenbach (1992) showed that TNP-ATP has similar effects on the steady state activity of CcO as ATP. However, certain other characteristics of the interaction were different between TNP-ATP and ATP. The binding of TNP-ATP to CcO from the matrix was 1000-fold stronger (K_d = 1.6 μM); and the binding stoichiometry was reported to be 2:1, where it appears to be 1:1 with ATP. In contrast, it was not possible to ascertain the number of binding sites on the cytosolic side because no saturation of the spectral change was observed with TNP-ATP in experiments with reconstituted CcO. The significant tighter binding of TNP-ATP to the allosteric effector site means, of course, that the allosteric interaction could be studied at effector concentrations that do not influence the docking between cytochrome c and CcO by ionic strength effects or interaction with cytochrome c.

The purpose of the present study is to define the interaction of TNP-aTP with CcO at the allosteric effector site more precisely so that the origin of the disparity in the association between ATP and TNP-aTP can be delineated. Toward this goal, we have compared the partitioning of TNP-ATP, 2' (or 3')-O-(2,4,6-trinitrophenyl) adenosine 5'-monophosphate (TNP-AMP), and ATP into various detergents commonly employed to solubilize CcO (Tween-80, Triton X-100, Brij 35, and lauryl-D-maltosikde), as well as their binding to various CcO-detergent micelles by the fluorescence titration and rapid filtration methods. Fluorescence titrations have also been undertaken at different temperatures in order to obtain estimates of the thermodynamic parameters associated with the binding, Both the interaction of TNP-ATP to detergent and CcO-detergent micelles show similar detergent dependences whereas the binding of ATP does not, suggesting that interaction of the hydrophobic trinitrophenyl moiety with the detergent contributes predominately to the free energy of binding of TNP-ATP to the CcO-detergent micelles. On the basis of these results, we conclude

that TNP-aTP binds to the allosteric site of CcO via the triphosphate linkage with the hydrophobic TNP group embedded in the detergent. The stoichiometry of the association of TNP-ATP with CcO was determined to 1:1 by rapid filtration.

MATERIALS AND METHODS:

Materials. Horse heart cytochrome c (grade VI), ATP (grade I disodium salt), 8-azido ATP (grade I disodium salt), ADP (grade I sodium salt), Tween-80 (grade II), Brij 35 and Triton X-100 were purchased from Sigma Chemical Co. (San Louis, MO). TNP-ATP and TNP-AMP were obtained from Molecular Probe (Eugene, OR) and 8-azido-[γ-^{32}P] ATP from ICN (Costa Mesa, CA). Lauryl-D-maltoside was from Fluka (Ronkonkoma, NY).

Cytochrome c oxidase was isolated and purified from bovine heart mitochondria by the method of Hartzell and Beinert (1974). The enzyme preparation was stored at -78°C before use. Enzyme concentrations were determined from $DA_{red\text{-}ox}$ at 605 nm using an extinction coefficient of 24 mM^{-1} (Van Gelder, 1966).

Fluorescence measurements· All fluoresence titration's of TNP-ATP (TNP-AMP) with CcO and with various detergents were performed using a SLM 4800 spectrofluorometer equipped with a regulated water circulating system and bath for precise temperature control. The fluorophore-TNP-ATP (TNP-AMP) was excited at 450 nm instead of 408 nm to avoid interference from the strong absorption of CcO at 410 nm. The fluorescence emission was scanned from 500 nm to 700 nm. The output from the SLM4800 spectrofluorometer was interfaced to a computer for data manipulation and analysis.

Fluorescence titration of TNP-ATP (or TNP-AMP) with detergent. 8.7 μM TNP-ATP (or 12.4 μM TNP-AMP) in 10 mM phosphate buffer, pH 7.4 was titrated with Tween-80-80 from a 20 %

stock solution. The fluorescence of TNP-ATP (TNP-ATP) was measured as the concentration of detergent was increased from 0.1 % to 1.2 %. These titrations were performed at both 3°C and 20°C. The binding constant of TNP-ATP (TNP-AMP) with Tween-80 was calculated by fitting the titration curve to equation 1.

$$F = F^{\circ}(C_T - C_D C_T K_1/(1 - C_D K_1)) + F^{\circ'}(C_D C_T K_1/(1 - C_D K_1)) \quad (1)$$

in which F is the observed fluorescence intensity of the TNP-ATP solution, F° and $F^{\circ'}$ are relative fluorescence intensity of the TNP-ATP (TNP-AMP) in solution and in detergent respectively; C_D is the effective detergent concentration and C_T is total or stoichiometric TNP-ATP concentration. C_D was determined from the formular weight of the detergent. We have assumed that 12 detergent molecules (this number is estimated from the stoichiometry of TNP-ATP binding to Lauryl-D-maltoside using rapid filtration method) are required for the binding of a TNP-ATP molecule. DG° for the partitioning of the TNP-ATP (TNP-AMP) into the detergent micelles was calculated from K_1 ($\Delta G = -RTlnK_1$)

Finally, ΔH° and ΔS° were estimated from the temperature dependence of K_1 assuming that ΔH° and ΔS° are only slowing varying functions of the temperature.

Fluorescence titration of TNP-ATP (or TNP-AMP) with CcO micelles. 16.9 μM of TNP-ATP (or 6.8 μM of TNP-AMP) was titrated with CcO in 10 mM phosphate buffer pH 7.4, 0.1 % Tween-80. The concentration of CcO was various from 0.9 to 9 μM and the fluorescence of the TNP-ATP was recorded as the concentration of CcO was increased.

Fluorescence titration of CcO micelles with TNP-ATP. 0, 1.8, or 3.6 μM of CcO in Tween-80 micelles was titrated with TNP-ATP in 10 mM phosphate buffer pH 7.4, 0.1 % Tween-80. The fluorescence of TNP-ATP was recorded as its concentration was varied. A control containing no CcO was used to correct for the inner filter effect of the TNP-ATP itself

If F^o is the relative fluorescence intensity of TNP-ATP in buffer, and F_1 is the background fluorescence,

$$F = (F^oC_T + F_1)\, exp(-M_1\, C_T) \quad (2)$$

where the $(F^oC_T+F_1)$ term corresponds to the increase of fluorescence intensity upon increasing the fluorophore concentration, and the $exp(-M_1 \cdot C_T)$ term corresponds to the inner filter effect. M_1 is a constant which is set by the instrument and the extinction coefficient of TNP-ATP at 450 nm, the wavelength of excitation in the fluorescence measurements. By fitting the fluorescence intensity versus the concentration of TNP-ATP in the control experiment with equation 2. M_1 was determined and applied to correct for the inner filter effect in the titration of CcO with TNP-ATP, as shown in equation 3.

$$F = (F^oC_T + F^{o''}\,[TNP\text{-}ATP \cdot CcO] + F_1)\, exp(-M_1\, C_T) exp(-M_2\, C_{CcO}) \quad (3)$$

in which C_T and C_{CcO} are the total concentration of TNP-ATP and CcO, respectively. The term $F^{o''}$[TNP-ATP·CcO] corresponds to the fluorescence intensity increase from the formation of TNP-ATP·CcO complex, $F^{o''}$ is fluorescence intensity increase per unit TNP-ATP·CcO complex formation. The terms, $exp(-M_1C_T)$ and $exp(-M_2C_{CcO})$ correspond to the inner filter effect of TNP-ATP and CcO to the fluorescence measurement ($M_2 = M_1\varepsilon_{CcO}/\varepsilon_{TNP}$, where ε_{TNP} and ε_{CcO} denote the extintion coefficients of TNP-ATP and CcO at 450 nm, respectively). [TNP-ATP·CcO] is determined by equation 4.

$$[TNP\text{-}ATP \cdot CcO] = ((K_2\, C_T + K_2\, C_{CcO} + 1) - ((K_2\, C_T + K_2\, C_{CcO} + 1)^2 - 4\, K_2^2\, C_T\, C_{CcO})^{0.5} / (2\, K_2) \quad (4)$$

Assuming the following equilibrium

TNP-ATP + CcO <==> TNP-ATP·CcO

K_2 is the binding constant between TNP-ATP and CcO. By fitting the fluorescence titration data simultaneously to equations 3 and 4, the binding constant between CcO and TNP-ATP could be determined.

Temperature dependence of the fluorescence of TNP-ATP in buffer with or without detergent, or CcO. Solutions containing 16.9 μM of TNP-ATP in 10 mM phosphate buffer, pH 7.0, with or without 1 % Tween-80, or in 10 mM phosphate buffer, 0.1% Tween-80, pH 7.4, with 9 μM CcO were prepared and the fluorescence of TNP-ATP was recorded as a function of the temperature from 3°C to 36°C. The solution without detergent and CcO was used as a control to correct for the decrease in quantum yield of the fluorophore with increasing temperature. The temperature dependence of the fluorescence of TNP-ATP in detergent or upon complexation with CcO could be used to determine ΔH^o and ΔS^o for the binding of TNP-ATP to the detergent and CcO, after correction for the reduced quantum yield at higher temperatures. ΔH^o and ΔS^o for the binding of TNP-ATP with Tween-80 were obtained by fitting the temperature dependence curves with equation 5, which is similar to equation 1 with some simplification.

$$F = C_T / (K_1 C_D + 1) (F^o + K_1 C_D F^{o\prime}) \quad (5)$$

and equation 6

$$K_1 = exp(-(\Delta H^o - T\Delta S^o) / (RT)) \quad (8)$$

For fitting the temperature dependence of TNP-ATP fluorescence in the presence of CcO, equation 3 was used in combination with equation 6.

Determination of the ATP and TNP-ATP detergent binding affinity by the rapid filtration method. The ATP or TNP-ATP were incubated in 10 mM phosphate buffer, pH 7.4, with or without detergent for 15 min at 4°C (other conditions are specified in Tables). The sample was then centrifuged in a Centricon 10 (Amicon Division, Beverly, MA) in a Sorvall Instrument RC5C centrifuge (Sorvall Instrument, Dupont) at a speed of 3,500 for 15 min at 3°C. After centrifugation, samples of the filtrate and the original solution were analyzed for the concentration of ATP or TNP-ATP by UV-VIS spectra according to the extinction coefficient of ATP at 260 nm (17 mM^{-1}) and TNP-ATP at 408 nm (26 mM^{-1}). The binding constant K_1 was estimated from the rapid filtration data in conjunction with equation 9.

$$K_1 = [TNP\text{-}ATP \cdot D] / ([TNP\text{-}ATP] [C_D]) \quad (9)$$

[TNP-ATP] is the concentration of free TNP-ATP in the solution at equilibrium, estimated from the TNP-ATP concentration in the filtrate; [TNP-ATP·D] is the concentration of detergent bound TNP-ATP, which was estimated from the concentration of TNP-ATP in the original buffer after subtracting that of the filtrate. [C_D] is free effective detergent concentration in buffer at equilibrium, and was estimated by subtracting [TNP-ATP·D] from the total effective detergent concentration.

Determination of the binding stoichiometry and binding affinity of TNP-ATP (ATP) to CcO by rapid filtration. The apparent binding stoichiometry and binding affinity of TNP-ATP (ATP) to native CcO was determined by centrifuging a preequilibrated 2 ml solution of TNP-ATP or ATP with 10 μM of native or 8-azido ATP modified CcO at 5 mM Tris buffer with detergent (specified in Tables) pH 7.4, (some of the buffer contain 100 mM KCl), in a centricon-100 microconcentrator, which retains the CcO but allows free ATP to pass the membrane, for 20 min at 3°C. Other conditions were specified in the Tables. After the centrifugation, the concentration of TNP-ATP, ATP in the filtrate and the original solution together with that of CcO in the original solution were determined by UV-Visible spectra.

Determination of the binding constant of cytochrome c to CcO by the rapid filtration method. To determine the binding constants of the cytochrome c to CcO under various conditions, a preequilibrated solution of 10 μM of cytochrome c with 10 μM of CcO in 5 mM Tris buffer, pH 7.4 (low salt condition) or 5 mM Tris buffer with 100 mM KCl, pH 7.4 (high salt condition) was centrifuged in a centricon-100, which retains CcO but allows free cytochrome c to pass the membrane, for 10 min at 3°C. ATP and TNP-ATP were also present in some of the experiments. 8-azido-ATP-modified CcO was also used in place of CcO in some experiments. Other conditions are specified in the Tables. After the centrifugation, the concentration of cytochrome c in the filtrate and the original solution together with that of CcO in the original solution were determined by UV-Visible spectrum.

Since the filtrate volume was about 20 % of the total volume, the species separated was assumed to reflect the concentration of the species in equilibrium in the original solution. Therefore, in experiments on the binding of native CcO with cytochrome c, we have

$[S] = C_S^F$

$[E \cdot S] = C_S^O - C_S^F$

$[E] = C_E^O - [E \cdot S]$

in which [S] is the concentration of free substrate (cytochrome c), [E] is the concentration of free enzyme (CcO) and [E·S] is the concentration of the enzyme substrate complex. C_S^F and C_S^O is the concentration of substrate in the filtrate and original solution respectively, C_E^O is the concentration of enzyme in the original solution.

Modification of CcO with 8-azido-ATP. CcO was diluted to a final concentration of 15μM in 10 mM Hepes buffer, pH7.4, 0.1 % Lauryl-D-maltoside. 3 mM of 8-azido-ATP was added to the samples and incubated for 20 min at 4°C. Then the mixture was illuminated with a Mighty Bright UV source (Hoefer Scientific instrument, San Francisco) in a 20 ml vial for one hour. To remove the unreacted 8-azido ATP, ADP and reaction side products, the samples were then loaded onto a DEAE-cellulose column and eluted with a linear gradient of 0-100 mM of NaCl with 5 mM phosphate and 0.1 % Lauryl-D-maltoside buffer, pH 7.4. The CcO fraction was collected from the column, and then washed with 5 mM Tris buffer containing 100 mM KCl, 0.1 % Lauryl-D-maltoside, pH7.0 with Centricon 100 miniconcentrators. The modified CcO was stored at -78°C until use.

Labeling of CcO with 8-azido-[γ-^{32}P] ATP. The labeling of CcO with 8-azido-[γ-^{32}P] ATP was done according to the method of Montecucco *et al.* (1986) with some modification. CcO was diluted to 15μM in 10 mM Hepes buffer, pH 7.4, 0.1 % Lauryl-D-maltoside. 3 mM ATP (or 30 μM of TNP-ATP/EDTA/EGTA) may also present in some of the samples. 8-azido-[γ-^{32}P] ATP was added and incubated for 20 min at 4°C. The mixture (300 μL) was then illuminated with a

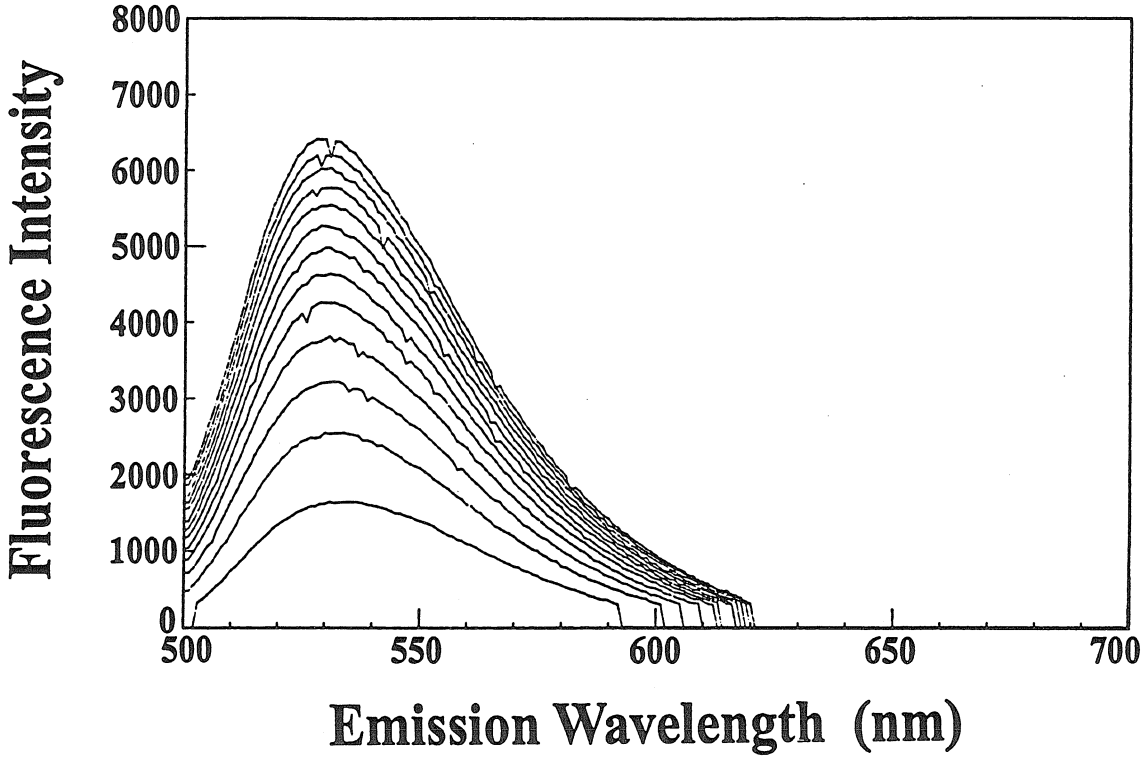
long wavelength u. v. lamp (Ultraviolet Products, San Gabriel, CA) for 20 min at 4°C. Following irradiation the samples were layered on 10 % (w/v) sucrose/10 mM Hepes buffer, pH 7.4 and centrifuged at 240,000 g for 6 hr in a Beckman Model L5-65 ultracentrifuge with a 60 Ti fixed angle rotor. The pellets were resuspended and subjected to electrophoresis in accordance with Lin *et al* (1993). The gels were stained with Coomassie Brilliant Blue, destained, dried and exposed to Kadak X-Omat at -80°C.

Steady state activity of CcO in the presence of ATP and TNP-ATP. The steady state activity of CcO was assayed in a buffer of 25mM Tris, pH 7.4, 0.05 % Lauryl-D-maltoside with or without 3mM ATP (or 30 μM TNP-ATP). The CcO concentration was 3 nM and the cytochrome c concentration was varied from 16 to 0.01 μM. The data are presented in Eadie-Hofstee plots. The activity is expressed as the molecular turnover number (TN = mole cytochrome c sec^{-1} permole CcO, at 20°C.

RESULTS:

Partitioning of TNP-ATP and TNP-AMP into Tween-80. The partitioning of TNP-ATP into detergent Triton X-100 micelles has been described by Tummino and Gafni (1992). Following their lead, we have investigated the behavior of the TNP-ATP in various detergents related to our investigation of the interaction between TNP-ATP and CcO-detergent particles, using various methods. In the fluorescence method, where we have exploited the increase of fluorescence of TNP-ATP accompanying the transfer of the fluorophore from aqueous buffer to a more hydrophobic environment, the effect of detergent concentration on the fluorescence of TNP-ATP as the detergent Tween-80 is increased from 0.1 to 1.2%, is shown in Figure VI.1. Accompanying the increase of fluorescence, the fluorescence maximum was also shifted from 535 nm to about 528 nm, as reported previously by Hiratsuka (1982). Figure VI.2 summarized the corresponding titration curve of TNP-ATP against Tween-80, where the fluorescence intensity I_{max} of TNP-ATP

Figure VI.1a and b. The effect of detergent concentration (Tween-80) on the fluorescence spectrum of TNP-ATP. Excitation wavelength, 550 nm. The bottom spectrum was obtained from a 8.7 μM TNP-ATP solution in 10 mM phosphate, pH 7.4, with 0.1 % Tween-80. As the concentration of Tween-80 was increased from 0.1 % to 1.2 % by 0.1 % increment each step, the fluorescence spectrum was measured. Stepwise increase of fluorescence intensity was observed from the titration as concentration of detergent increased. Figure VI.1a and b were measured at 3°C and 20°C, respectively.


Fluorescence Intensity
8000
7000
6000
5000
4000
3000
2000
1000
0
500
550
600
650
700
Emission Wavelength (nm)

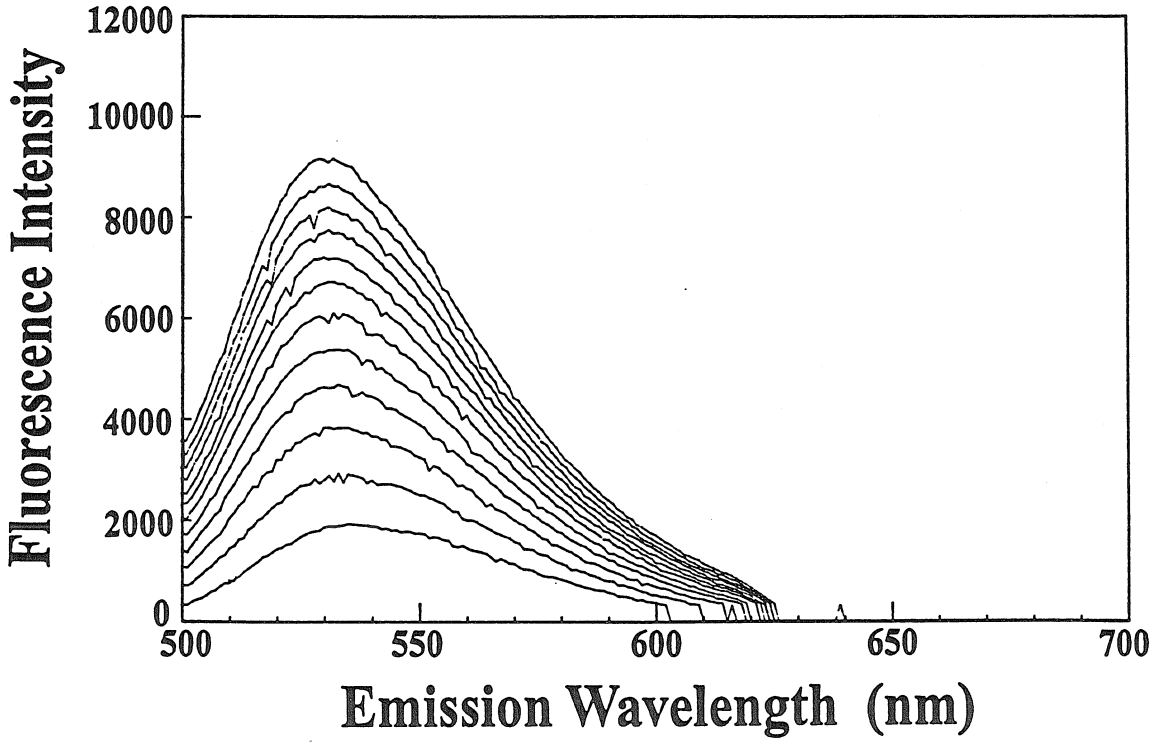
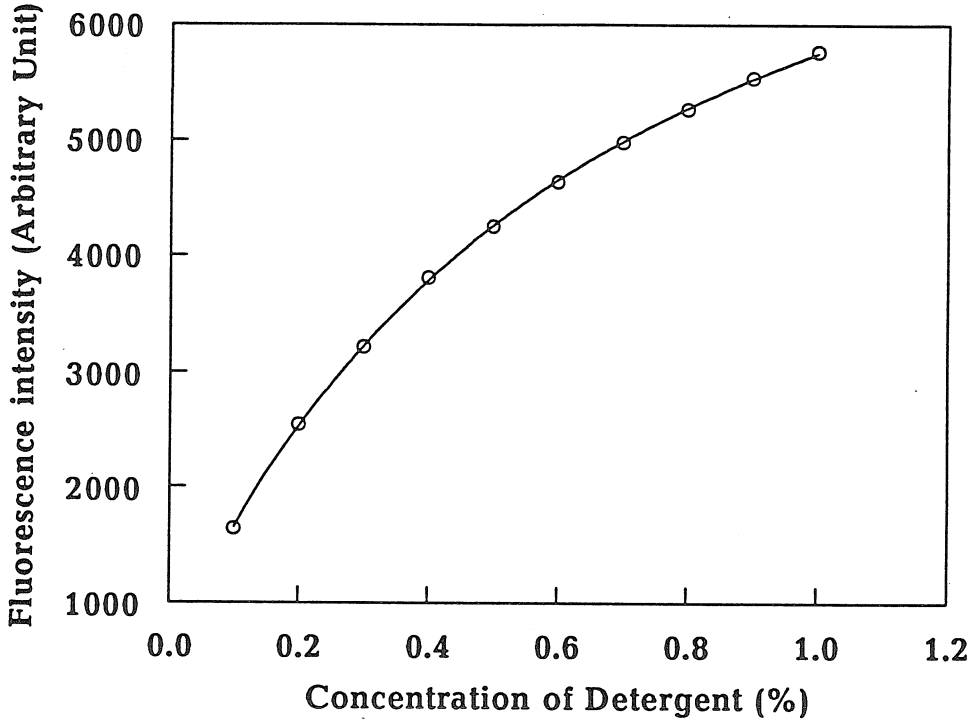

Fluorescence Intensity
12000
10000
8000
6000
4000
2000
0
500
550
600
650
700
Emission Wavelength (nm)

Figure VI.2a and b. The fluorescence titration curve of TNP-ATP with detergent. The maximal of fluorescence intensity of the Figure VI.1a and b was plot against the concentration of detergent. The corresponding titration curves were called Figure VI.2a and b. The solid line between data points represent the data fitting of the Figure VI.2a and b with Eq. 1.


Fluorescence intensity (Arbitrary Unit)
6000
5000
4000
3000
2000
1000
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Concentration of Detergent (%)

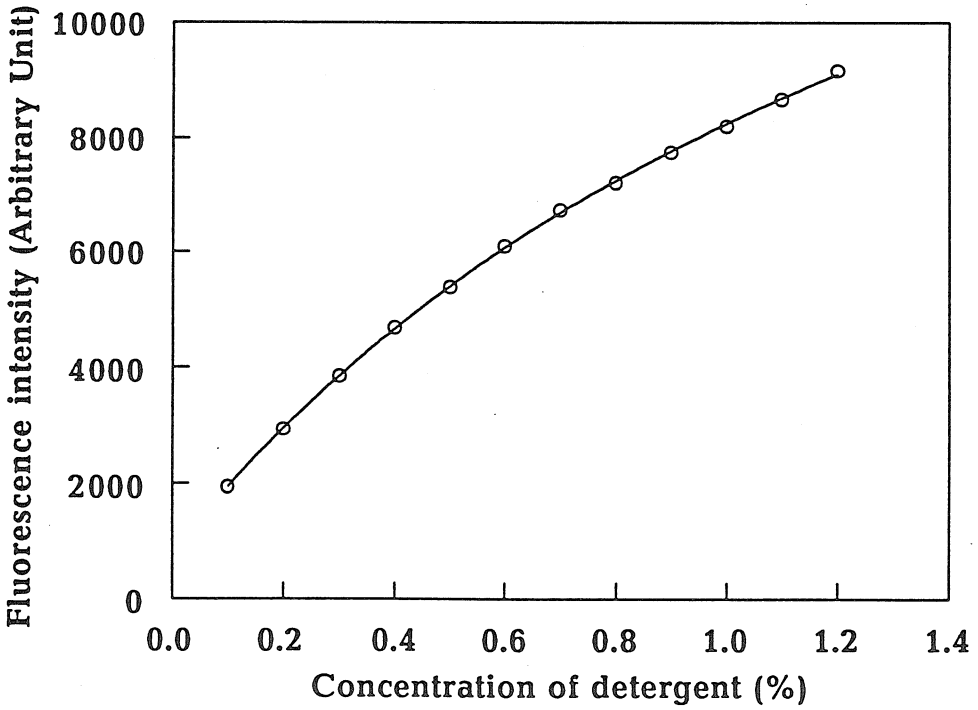

Fluorescence intensity (Arbitrary Unit)
10000
8000
6000
4000
2000
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Concentration of detergent (%)

Table 1. Equilibrium parameters of TNP-ATP and TNP-AMP binding to Tween-80 from fluorescence titration of TNP-AXP with detergent and temperature dependence of fluorescence*

	K (mM^{-1})	ΔG^o (KJ/mol)	ΔH^o (KJ/mol)	ΔS^o (J/mol/K)
TNP-ATP at 20°C[1]	0.79	-16	-35†	-64†
TNP-ATP at 3°C[1]	1.24	-17		
TNP-AMP at 20°C[1]	3.68	-20	-51†	-106†
TNP-AMP at 3°C[1]	13.4	-22		
TNP-ATP at 20°C[2]	0.30¶	-14¶	-33	-65
TNP-ATP at 3°C[2]	0.81¶	-15¶		

[1] Obtained from data fitting of fluorescence titration of TNP-AXP with detergent

[2] Obtained from data fitting of temperature dependence of TNP-ATP fluorescence in 1 % Tween-80

† Calculated from ΔG of TNP-ATP binding to detergent at different temperature according to Eq. (3)

¶ Calculated from ΔH and ΔS of TNP-ATP from data fitting of temperature dependence of TNP-ATP fluorescence in 1 % Tween-80 according to Eq. (3) and (2)

* The equilibrium parameters were calculated by assuming 12 molecules are required for binding one molecule of TNP-ATP

has been plotted against the concentration of the detergent. When these data were fitted to equation 1, binding constant of TNP-ATP to Tween-80 was determined to be 0.79 mM^{-1} and 1.24 mM^{-1} at 20°C and 3°C, respectively (Table 1). Throughout this paper, we have assumed that 12 molecules of detergent are required to bind one molecule of TNP-ATP. This number was estimated from the stoichiometry of binding of the TNP-ATP to various detergent as determined by rapid filtration. ΔH and ΔS for the binding of TNP-ATP to Tween-80 could be estimated from the temperature dependence of K_1. These results are summarized in Table 1.

Aside from Tween-80 we have studied the partitioning of TNP-ATP in Brij 35 and Lauryl-D-maltoside. Similar effect on the fluorescence intensity of TNP-ATP as Tween-80 was observed with Brij 35 as the detergent concentration was increased from 0 to 1 % (Figure VI.3). Lauryl-D-maltoside was observed to increase the intensity of fluorescence in a greater extent than Brij-35 and Tween-80, at low concentrations (0.1%) (data no shown). These results suggest higher affinity for the partition between TNP-ATP and Lauryl-D-maltoside than that with Tween-80 or Brij 35.

The partitioning of TNP-AMP into Tween-80 was also studied in the same manner. The fluorescence titration curve of TNP-AMP is shown in Figure VI.4a and b at 3°C and 20°C, respectively. TNP-AMP shows similar detergent behavior as its triphosphate form, although its fluorescence intensity reached saturation at a lower detergent concentration than for TNP-ATP. Fitting of the data to equation 1 give a binding constant of 3.68 mM^{-1} and 13.4 mM^{-1} for the TNP-AMP binding to Tween-80 micelles at 20°C and 3°C, respectively. The thermodynamic parameters obtained from the data fitting are summarized in Table 1. Apparently, the binding affinity for TNP-AMP to Tween-80 is higher than that of TNP-ATP. These results suggest that the partitioning of TNP-AXP to detergent is predominantly hydrophobic in nature.

Temperature dependence of the partitioning of TNP-ATP to Tween-80. Figure VI.5a. shown the temperature dependence of the fluorescence of TNP-ATP in buffer without detergent. The relative

Figure VI.3. The effect of the concentration of Brij-35 on the fluorescence spectrum of TNP-ATP. The bottom spectrum was obtained from a 8.7 μM TNP-ATP solution in 10 mM phosphate, pH 7.4, at 3°C. As the concentration of Brij-35 was increased from 0 to 1.0 % by 0.2 % increment each step, the fluorescence spectrum was measured. Stepwise increase of fluorescence intensity was observed from the titration as concentration of detergent increase.

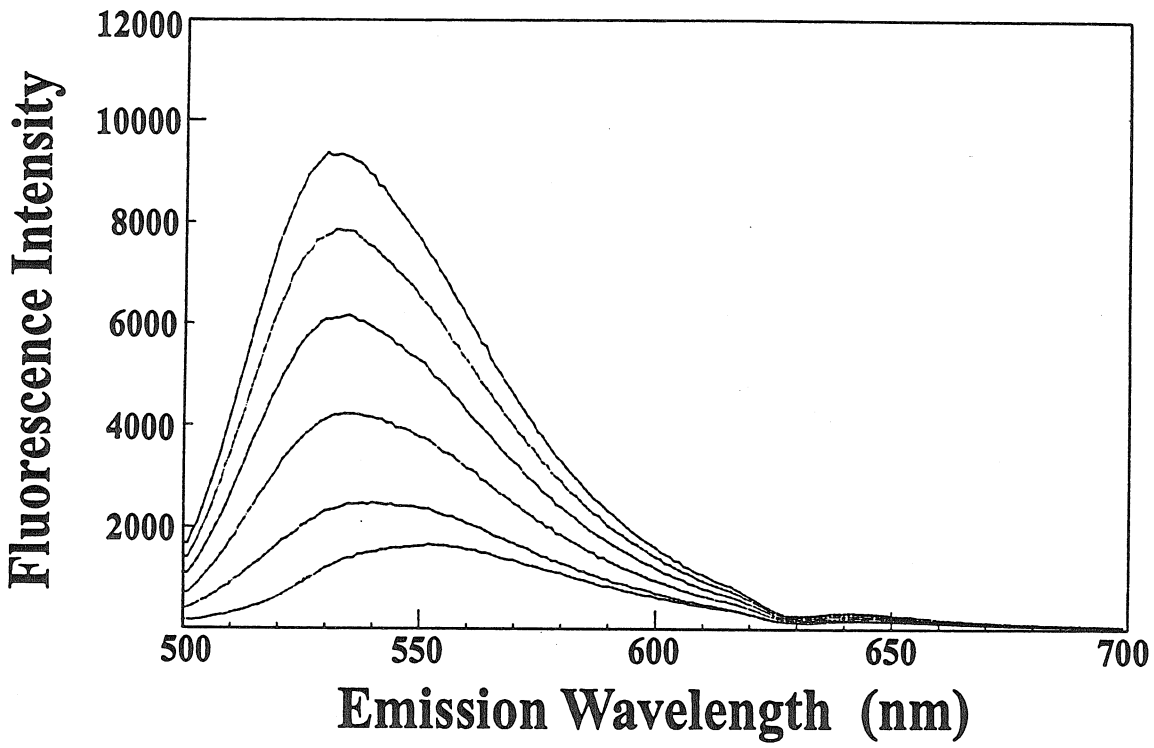
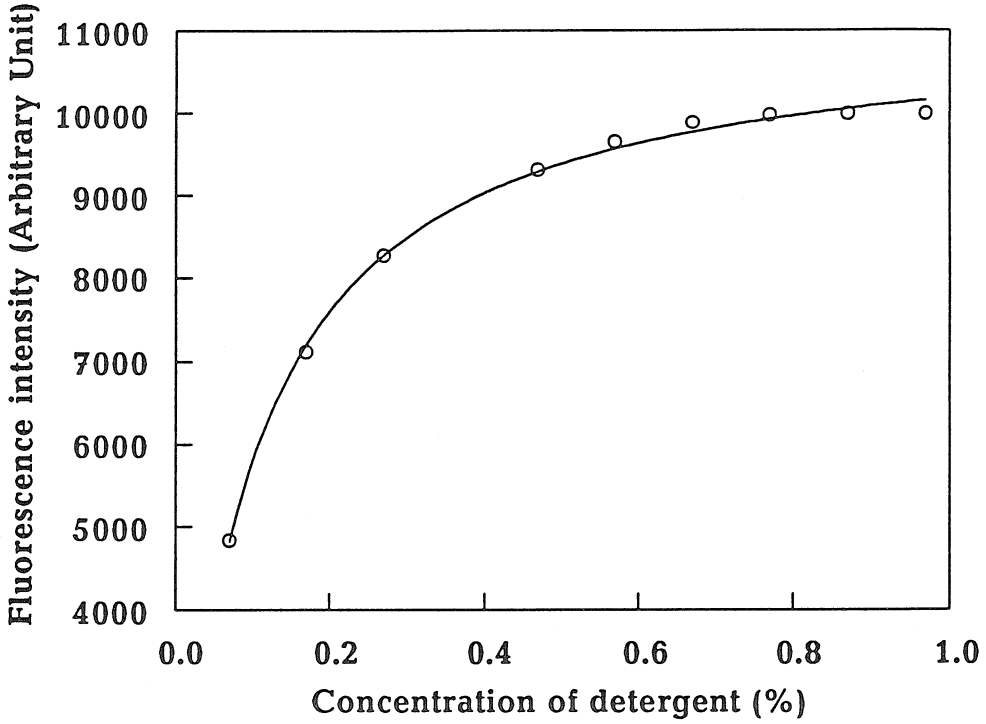

Fluorescence Intensity
12000
10000
8000
6000
4000
2000
500
550
600
650
700
Emission Wavelength (nm)

Figure VI.4a and b. The fluorescence titration curve of TNP-AMP with detergent. 12.4 μM of TNP-AMP was titrated with Tween-80. The detergent concentration was increase from 0.1 % to 1.0 % by 0.1 % increment each step, while the fluorescence spectra were recorded. The maximal of fluorescence intensity was, then, plotted against the concentration detergent. Titration of Figure VI.4a and b were done at 3°C and 20°C, respectively. Other conditions are same as Figure VI.1 and 2. The solid line between data points represent the data fitting of the Figure VI.4a and b with Eq. 1.


Fluorescence intensity (Arbitrary Unit)
11000
10000
9000
8000
7000
6000
5000
4000
0.0
0.2
0.4
0.6
0.8
1.0
Concentration of detergent (%)

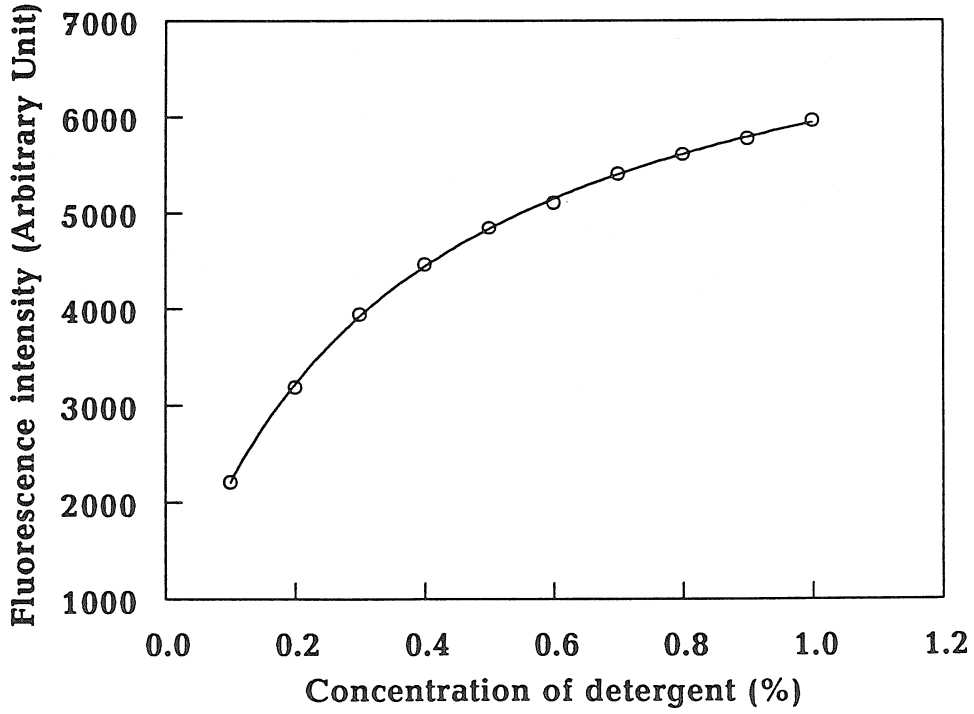
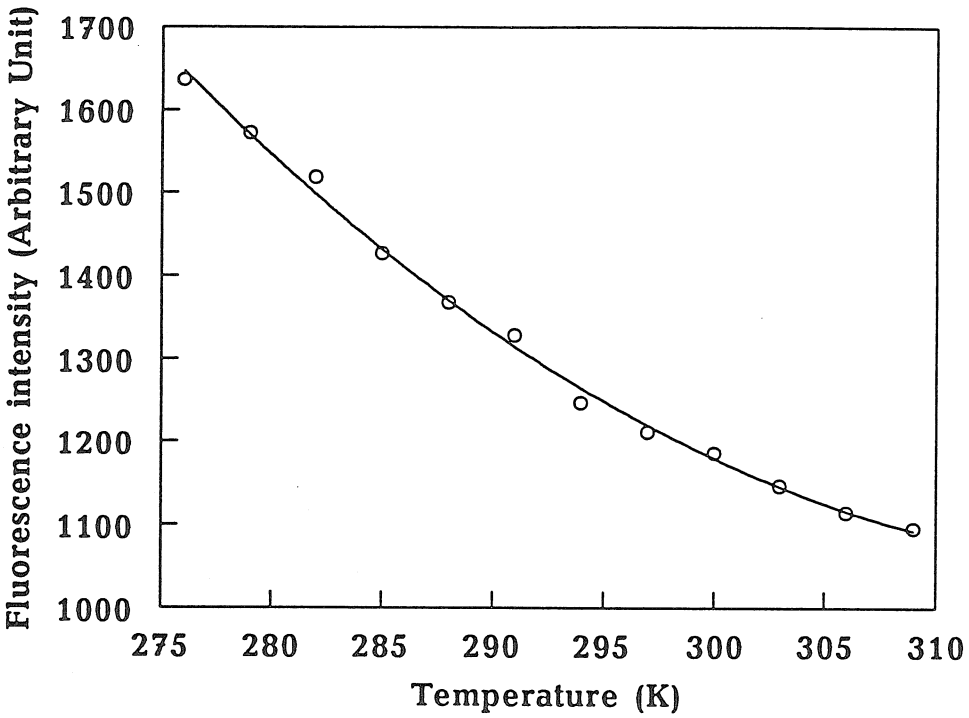
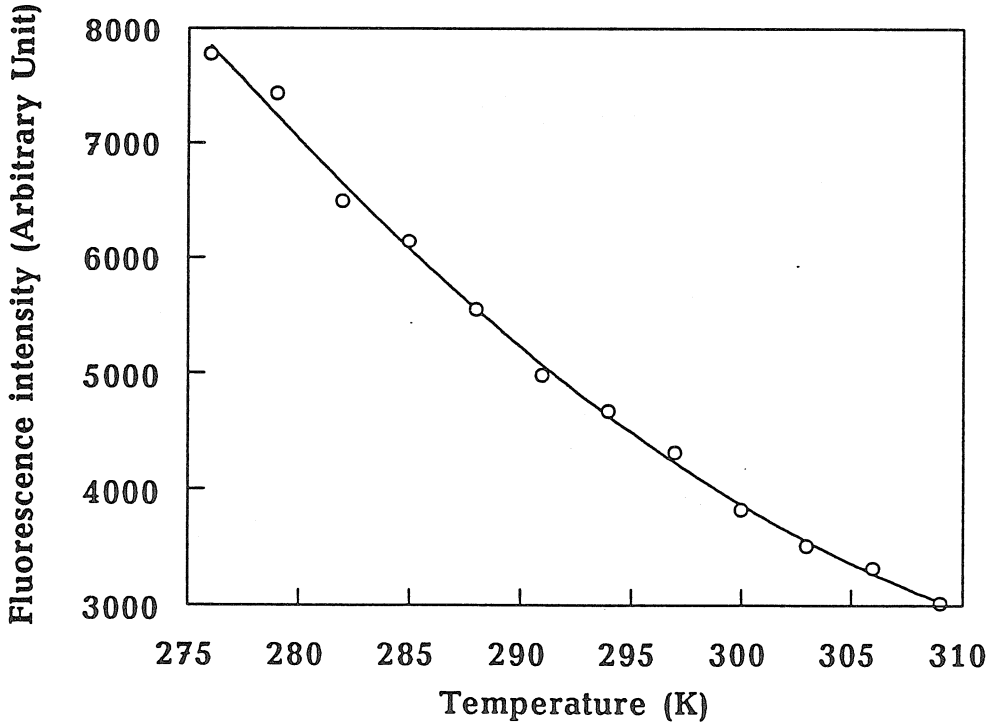

Fluorescence intensity (Arbitrary Unit)
7000
6000
5000
4000
3000
2000
1000
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Concentration of detergent (%)

Figure VI.5a and b. The effect of temperature on TNP-ATP fluorescence intensity with or without detergent. The fluorescence intensity of 16.9 μM of TNP-ATP was plotted against the temperature change from 3°C to 36°C, in 10 mM phosphate buffer, pH 7.4. (5a) control (without detergent); (5b) with 1% Tween-80 after correction of the temperature dependence of quantum yield from figure VI.5a. Solid line between data point represents the data fitting with Eq. 7 and 8.


Fluorescence intensity (Arbitrary Unit)
1700
1600
1500
1400
1300
1200
1100
1000
275
280
285
290
295
300
305
310
Temperature (K)


Fluorescence intensity (Arbitrary Unit)
8000
7000
6000
5000
4000
3000
275
280
285
290
295
300
305
310
Temperature (K)

intensity of the fluorescence was observed to decrease from 1636 to 1095 (33 % decrease) between 3°C and 36°C. This decrease of the fluorescence could be due to the reduction of quantum yield of the fluorophore at the higher temperature. Figure VI.5b. shows the temperature dependence of TNP-ATP in buffer with 1% Tween-80 after the correction of the reduction of the quantum yield with increasing temperature (Figure VI.5a). This temperature event is reversible, as the fluorescence intensity returns to its starting level, when the temperature is adjusted back to 3°C after the temperature has been increased to 36°C. Fitting of the data of Figure VI.5b to equation. 5 and 6 provided estimates of the ΔH and ΔS (Table 1) for binding of TNP-ATP to Tween-80 micelles. These results show good agreement with those obtained by the fluorescence titration method.

Affinity of different detergents for TNP-ATP as determined by the rapid filtration method. The rapid filtration method to determine the interaction between TNP-ATP and different detergents is similar to that used by Tummino and Gafni (1992) to study the partitioning of TNP-ATP to reduced Triton X-100 micelles. When the detergent buffer is forced through a membrane with Molecular Weight Cut-off (MWCO = 10 KDalton) smaller than the average size of detergent micelles (90 KDalton for some detergent micelles), the detergent micelles will be retained in the original buffer and the free substrate (TNP-ATP) will go through the membrane. This method allows us to directly observe the direct binding of any small molecule to detergent micelles and to calculate the binding constant.

Table 2 shows the binding constants of TNP-ATP to Tween-80, Brij 35, Triton X-100 and Lauryl-D-maltoside determined using this method. Lauryl-D-maltoside (K_1 = 24.9 mM^{-1}) was shown to have a higher affinity for TNP-ATP than Tween-80 (K_1 = 2.3 mM^{-1}), Brij-35 (K_1 = 2.7 mM^{-1}), and Triton X-100 (K_1 = 13.3 mM^{-1}). As expected, the affinity of various detergents for TNP-ATP varies from detergent to detergent. As reported previously Tummino and Gafni (1992), no partitioning of ATP into detergent was observed by the rapid filtration method (data no shown).

Table 2. Equilibrium parameters of TNP-ATP binding to different detergents by rapid filtration*

Detergent	K (mM^{-1})	ΔG^{o} (KJ/mol)
Tween-80	2.3	-18
Triton X-100	13.3	-22
Brij 35	2.7	-18
Lauryl-D-maltoside	24.9	-23

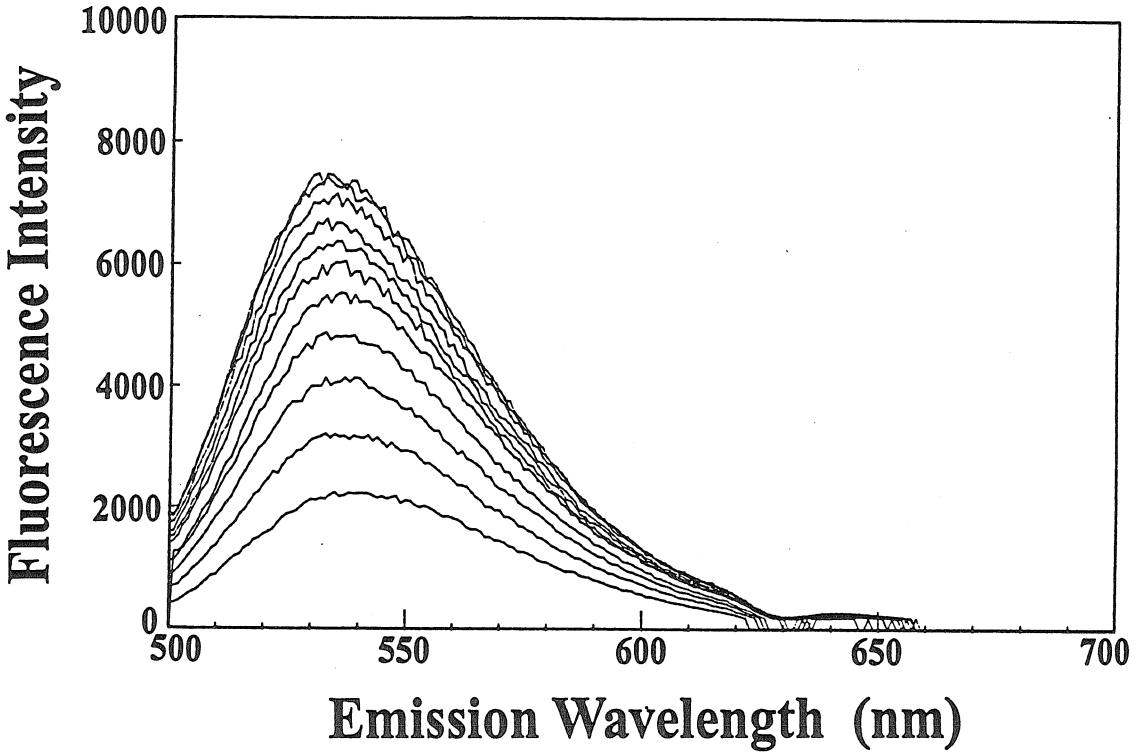
* Equilibrium parameters were calculated by assuming that 12 molecules of detergent are required for binding one molecule of TNP-ATP. Concentration of detergent used: [Tween-80] = 0.1 or 0.5 %; [Brij 35] = 0.1 or 0.5 %; [Triton X-100] = 0.1 or 0.5 %; [lauryl-D-maltoside] = 0.1 %. The results of Tween-80, Brij 35, and Triton X-100 are the average value from 0.1 and 0.5 % detergent experiments, which show good agreement.

The difference in the affinity for TNP-ATP and ATP by various detergents suggests the important role of the TNP group and hydrophobic interaction in the partitioning.

The binding of TNP-ATP to CcO by fluorescence titration. To study the binding between TNP-ATP and CcO, TNP-ATP was titrated with CcO at 3°C. The fluorescence intensity of TNP-ATP increases with the concentration of CcO, accompanied by a shift of the fluorescence maximum from 543 nm to 534 nm (Figure VI.6). As shown in Figure VI.7, CcO has a smaller effect on the fluorescence intensity of TNP-AMP. The different affinity of TNP-ATP and TNP-AMP towards CcO is opposite of that towards detergent, in which TNP-AMP has the higher affinity. It is, thus, evident that the binding of TNP-ATP to CcO is specific. Neither TNP group, nor the adenosine group is important in the binding to CcO. On the other hand, the triphosphate tail of TNP-ATP seems absolutely essential. We (Lin *et al.*, 1992) have previously proposed that the putative Mg^{2+} associated with subunit IV may provide the loci of interaction between ATP and CcO.

Figure VI.8 summarizes the titration of CcO with various concentration of TNP-ATP. The control without CcO is shown in Figure VI.8a. The fluorescence intensity of TNP-ATP does not increase in linearly with ATP concentration because of the inner filter effect. Fitting the data to equation 3 provides us with the inner filter effect parameter, which was in turn used to correct the CcO titration data for the inner filter effect. Figure VI.8b summarizes the titration of CcO (1.8 (A) and 3.6 μM (B)) with TNP-ATP. As expected, fluorescence intensity saturates at lower TNP-ATP concentration for 3.6 μM CcO than for 1.8 μM CcO. An apparent binding constant of 0.29 μM^{-1} (A) (or 0.25 μM^{-1} (B)) for the TNP-ATP was obtained from the data fitting of the titration curves with Equation 3 and 4. The K_d of TNP-ATP with CcO is in the μM range (3.4 or 4.0 μM), in reasonably close agreement to the value of previously reported by Reimann and Kadenbach (1992) (1.6μM) for CcO in Lauryl-D-maltoside. The detergent we used in the titration studies is Tween-80. The K_d value obtained from CcO in Lauryl-D-maltoside is expected to be higher than that in Tween-80, as shown later.

Figure VI.6a and b. The effect of CcO on the fluorescence spectrum of TNP-ATP. 16.9 μM of TNP-ATP was titrated with CcO in 10 mM phosphate buffer, pH 7.4 with 0.1 % Tween-80. The CcO concentration was increase from 0 to 9 μM by 0.9 μM increment each step. Figure VI.6a is the fluorescence spectrum of TNP-ATP, Figure VI.6b is the corresponding titration curve from Figure VI.6a. Other conditions are same as Figure VI.1 and 2.


Fluorescence Intensity
10000
8000
6000
4000
2000
0
500
550
600
650
700
Emission Wavelength (nm)

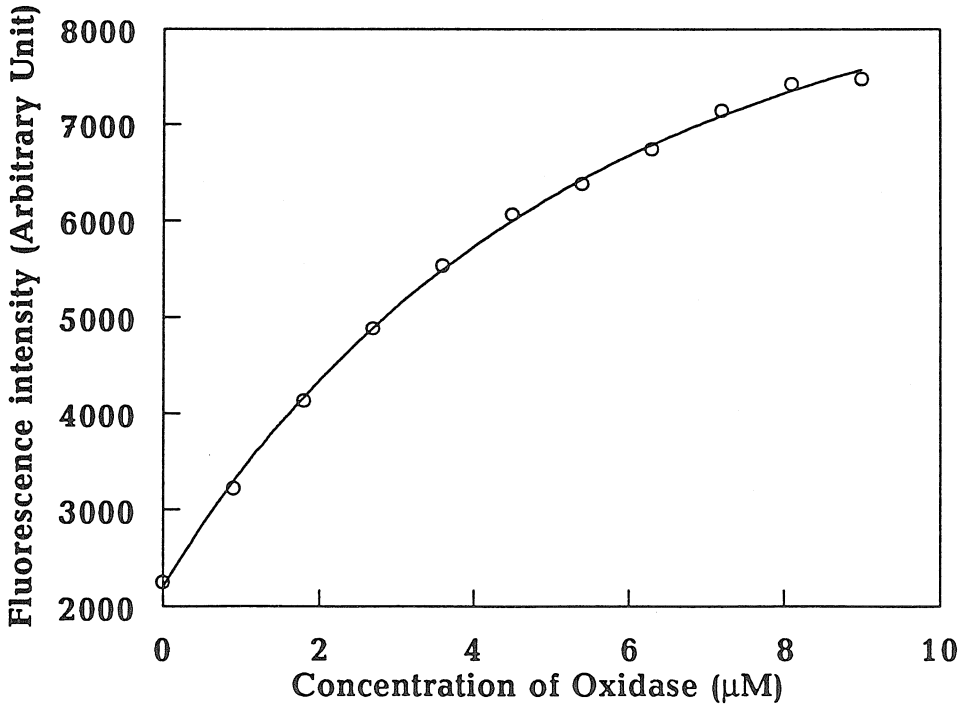

Fluorescence intensity (Arbitrary Unit)
8000
7000
6000
5000
4000
3000
2000
0
2
4
6
8
10
Concentration of Oxidase (μM)

Figure VI.7. The effect of CcO on the fluorescence spectrum of TNP-AMP. 6.8 μM of TNP-AMP was titrated with CcO in 10 mM phosphate buffer, pH 7.4 with 0.1 % Tween-80. The CcO concentration was increased from 0 to 9 μM by 1.8 μM increment each step. Other conditions are the same as Figure VI.1 and 2.

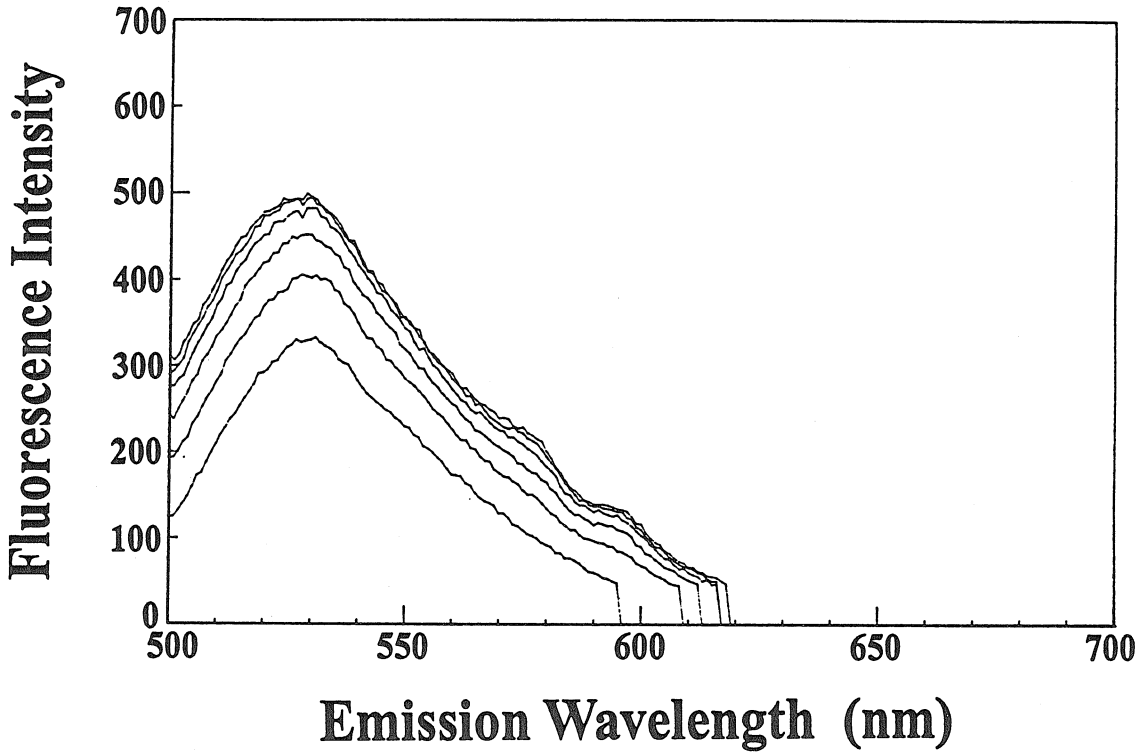
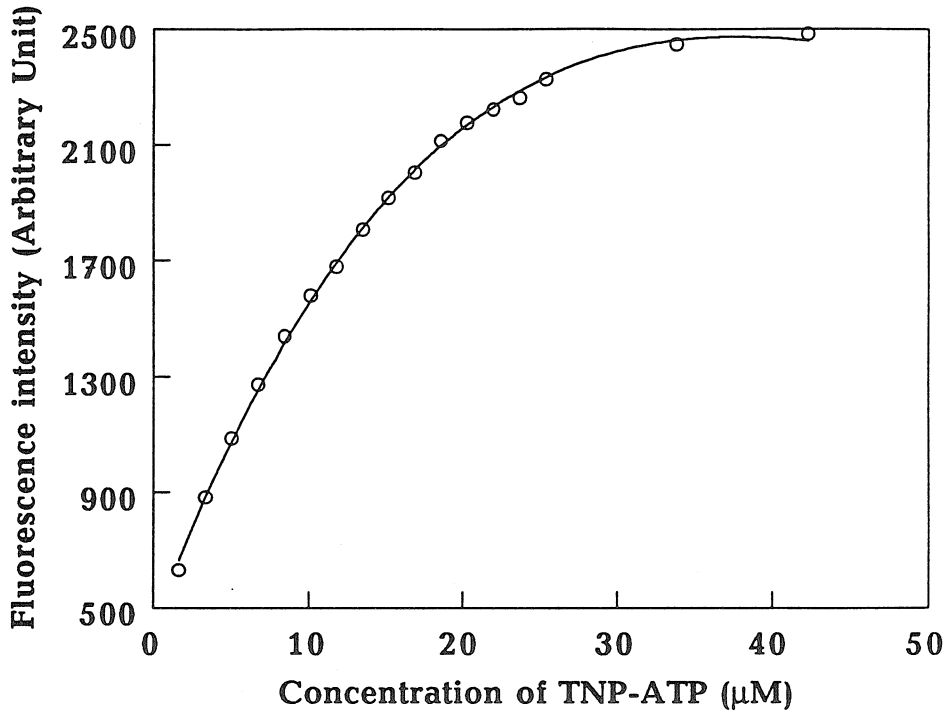
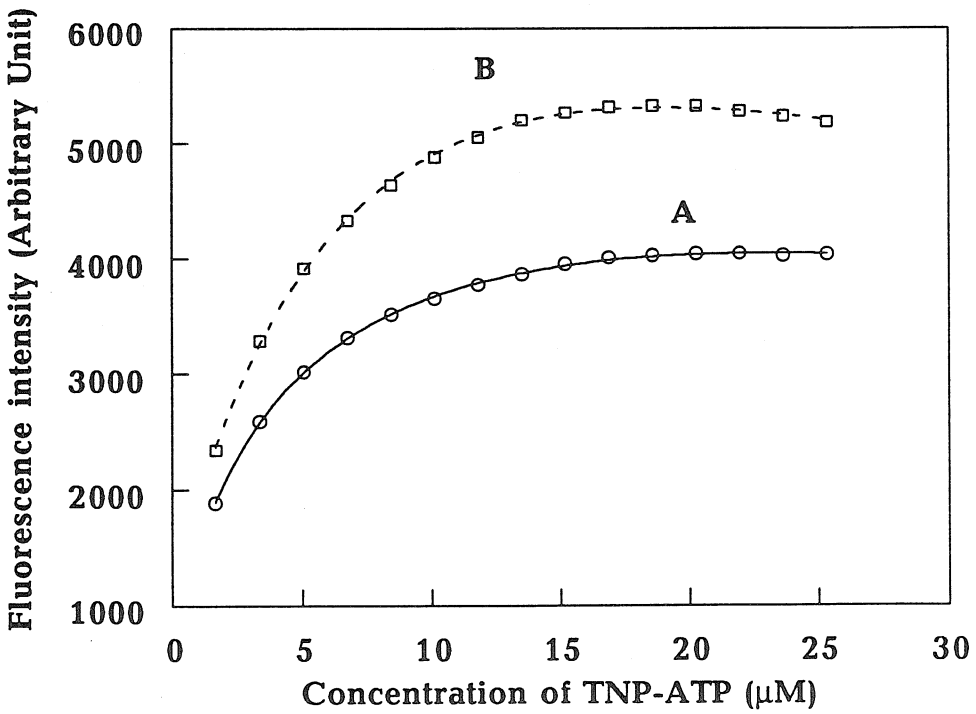

Fluorescence Intensity
700
600
500
400
300
200
100
0
500
550
600
650
700
Emission Wavelength (nm)

Figure VI.8a and b. The fluorescence titration curve of CcO with TNP-ATP. Figure VI.8a is the control experiment (without CcO). The maximal of fluorescence intensity was plotted against the concentration of TNP-ATP in 10 mM phosphate buffer, pH 7.4 with 0.1 % Tween-80, at 3°C. Figure VI.8b shows the fluorescence intensity against the concentration of TNP-ATP in 10 mM phosphate buffer, pH 7.4, 0.1 % Tween-80, with 1.8 μM CcO (A) or 3.6 μM CcO (B). The solid line between data points in Figure VI.8a represents the data fitting of the Figure VI.9a with Eq. 4, and the lines between data points in Figure VI.8b (A and B) represent the data fitting of the Figure VI.8b with Eq. 5 and 6.


Fluorescence intensity (Arbitrary Unit)
2500
2100
1700
1300
900
500
0
10
20
30
40
50
Concentration of TNP-ATP (μM)


Fluorescence intensity (Arbitrary Unit)
6000
5000
4000
3000
2000
1000
B
A
0
5
10
15
20
25
30
Concentration of TNP-ATP (μM)

Temperature dependence of TNP-ATP binding to CcO. Figure VI.9 shows the temperature dependence of TNP-ATP fluorescence in the presence of CcO, after correcting for the reduced quantum yield with temperature (Figure VI.5a). As the temperature is increased from 3°C to 36°C, the relative fluorescence intensity decreases. Eq. 3 and 6 were used to fit the temperature dependence of the fluorescence of TNP-ATP in the presence CcO. From these data, ΔH and ΔS for the interaction between TNP-ATP and CcO were estimated (ΔH = -51 $KJmol^{-1}$; ΔS = -72 $Jmol^{-1}K^{-1}$). The binding constant K and ΔG for TNP-ATP and CcO (at 3°C) were, then, calculated from ΔH and ΔS. These results are listed in Table 4.

Determination of the binding constant of TNP-ATP and ATP to CcO in different detergents by the rapid filtration method. The binding constants of TNP-ATP and ATP to CcO in different detergents was determined by the rapid filtration method. The Centricon 100, which has a MWCO of 100 K Dalton, permits the detergent micelles to pass the membrane but not the CcO. This will simplify our measurement to only the interaction of TNP-ATP and ATP with CcO.

Table 3 shows the results of these measurements. The binding of TNP-ATP to CcO varies from detergent to detergent. TNP-ATP binds much stronger to CcO in Lauryl-D-maltoside than in Tween-80.

ATP was also found to bind to CcO according to the rapid filtration method, but with a greatly reduced affinity and without detergent dependence. The standard ΔG differences between the TNP-ATP and ATP binding to CcO are -15 KJ/mol and -19 KJ/mol in Tween-80 and Lauryl-D-maltoside, respectively, which are close to the ΔG of TNP-ATP partitioning into Tween-80 (ΔG = -18 KJ/mol) and Lauryl-D maltoside (ΔG = -23 KJ/mol), respectively. The difference of ΔG for TNP-ATP binding to CcO in Lauryl-D-maltoside and Tween-80 ($\Delta(\Delta G)$ = -4 KJ/mol) is also close to the difference of ΔG for TNP-ATP binding to Lauryl-D-maltoside and Tween-80 ($\Delta(\Delta G)$ = -5

Table 3. Equilibrium parameters of TNP-ATP and ATP binding to CcO by rapid filtration*

Sample	K (mM^{-1})	ΔG^{o} (KJ/mol)
TNP-ATP with CcO in 0.1% Tween-80	260¶	-29
TNP-ATP with CcO in 0.1% Lauryl-D-maltoside	2140¶	-33
ATP with CcO in 0.1% Tween-80	0.47	-14
ATP with CcO in 0.1% Lauryl-D-maltoside	0.37	-14

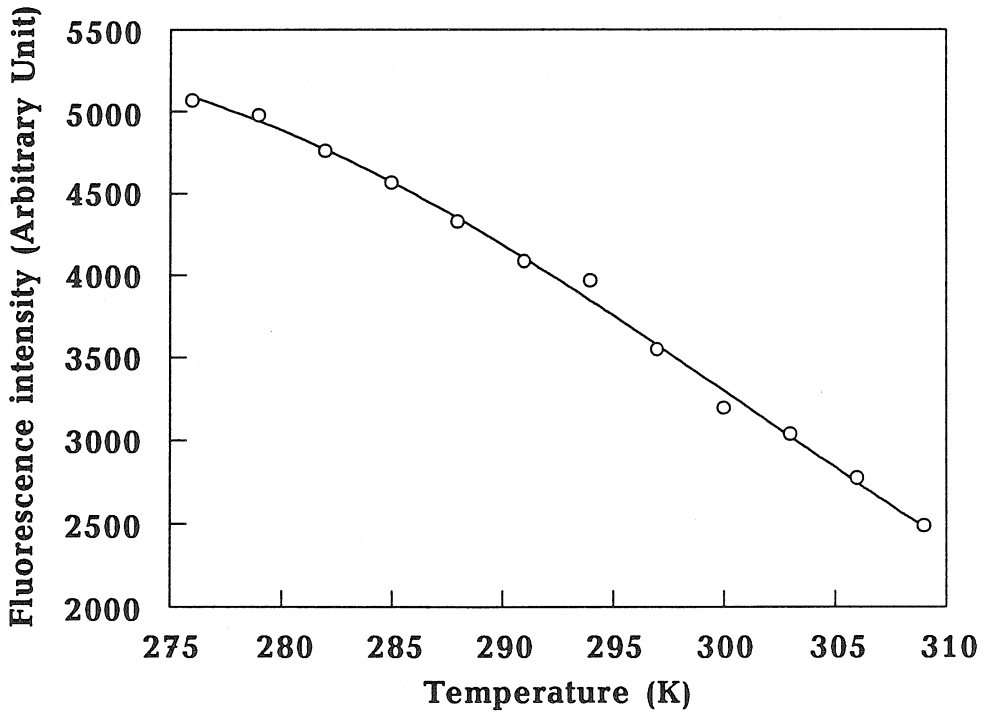
* concentration of CcO is 25 μM. In these experiments, the concentration of detergent is 0.1 or 0.05 % for Tween-80 and Lauryl-D-maltoside, respectively. TNP-ATP concentration is 18 μM and ATP concentration is 70 μM.
¶ to determine the binding constant between TNP-ATP and CcO in different detergents, the detergent effect was also taken into account. Free effective detergent concentration was calculated by $[S] = C_S^F - C_S^D$, in which C_S^F is the concentration of substrate in the filtrate, C_S^D is the concentration of detergent bound substrate. C_S^D is estimated from $C_S^D = (KC_DC_S^F)/(1+KC_D)$, in which C_D is the total effective detergent concentration, K is the binding constant for TNP-ATP to Lauryl-D-maltoside or Tween-80 from Table 2.

Table 4. The equilibrium parameters of TNP-ATP binding to CcO from fluorescence titration, rapid filtration and temperature dependence

Method	K (mM^{-1})	ΔG^{o} (KJ/mol)
Fluorescence titration	290	-29
Rapid filtration	260	-29
Temperature dependence	915¶	-32¶

¶ Calculated from ΔH = -51 $KJmol^{-1}$ and ΔS = -72 $Jmol^{-1}K^{-1}$ of TNP-ATP binding to CcO from data fitting of temperature dependence of TNP-ATP fluorescence in CcO according to Eq. (3) and (2)

Figure VI.9. The effect of temperature on TNP-ATP fluorescence intensity with CcO. The fluorescence intensity of 16.9 μM of TNP-ATP was plotted against the temperature change from 3°C to 36°C, in 10 mM phosphate buffer, pH 7.4, 0.1 % Tween-80 with 4.5 μM CcO. The solid line between data points represents the data fitting of Figure VI.9 with Eq. 5 and 8.


Fluorescence intensity (Arbitrary Unit)
5500
5000
4500
4000
3500
3000
2500
2000
275
280
285
290
295
300
305
310
Temperature (K)

KJ/mol). These indicate do suggest that the TNP group of TNP-ATP does contribute to the binding of TNP-ATP to CcO by interacting with detergent. This latter interaction account for the higher binding affinity as well as the observed detergent dependence.

The stoichiometry and specificity of TNP-ATP binding to CcO. To determine the stoichiometry of TNP-ATP binding to CcO, we have once again employed the 10 μM of CcO with 30 and 60 μM of TNP-ATP, at low ionic and high ionic strength (Table 5). No significant ionic strength effect was observed for the stoichiometry. Since the K_d for TNP-ATP binding at Tween-80 is around 0.5 μM^{-1}, 60 μM of TNP-ATP should saturate the binding. The results shown in Table 5 suggest 1:1 ratio for the TNP-ATP-CcO complex.

No competition of ATP or ADP with the binding of TNP-ATP has been observed by fluorescence (data no shown) or fast filtration (Table 6), even with concentration as high as 30 mM. On the contrary, modified CcO with 8-azido ATP do greatly decrease the binding of TNP-ATP, suggesting that the binding site of TNP-ATP has been greatly abolished by the cross-linked 8-azido-ATP. Thus, ATP and TNP-ATP must compete for the same site on CcO.

Effect of TNP-ATP on the binding between cytochrome c and CcO. The binding of TNP-ATP to CcO, as does 8-azido-ATP modification, greatly decreases the binding affinity between cytochrome c and CcO at low ionic strengths (Table 7). There is no simple way to determine the effect of ATP on the binding between cytochrome c and CcO at low ionic strength, since the concentration of ATP required for saturation of ATP binding (several mM) will usually result in high ionic strengths. At high ionic strengths, ATP, TNP-ATP and 8-azido ATP modification all exhibit similar, though seemingly minor, effects on the binding between cytochrome c and CcO.

Effects of TNP-ATP on steady state activity of CcO. Figure VI.10 shows the Eadie-Hofstee plots of CcO with or without ATP or TNP-ATP. TNP-ATP at a concentration of 30 μM behaves very

Table 5. Apparent binding stoichiometry of TNP-ATP to 10 μM CcO at low[a] and high[b] ionic strengths*

Sample	Apparent binding Stoichiometry ([TNP-ATP]:[CcO])
with 30 μM TNP-ATP at low ionic strengths	0.49
with 60 μM TNP-ATP at low ionic strengths	0.97
with 30 μM TNP-ATP at high ionic strengths	0.79
with 60 μM TNP-ATP at high ionic strengths	0.91

*5 mM of Tris buffer, 0.1 % Lauryl-D-maltoside, pH 7.4 was used.
[a] ionic strength is 5 mM.
[b] ionic strength is 105 mM.

Table 6. Apparent binding stoichiometry of TNP-ATP to native and 8-azido ATP modified CcO at various conditions*

Sample	Apparent binding Stoichiometry ([TNP-ATP]:[CcO])
native CcO	0.95
native CcO with the presence of 30 mM ATP	0.87
native CcO with the presence of 30 mM ADP	0.92
8-azido ATP modified CcO	0.36

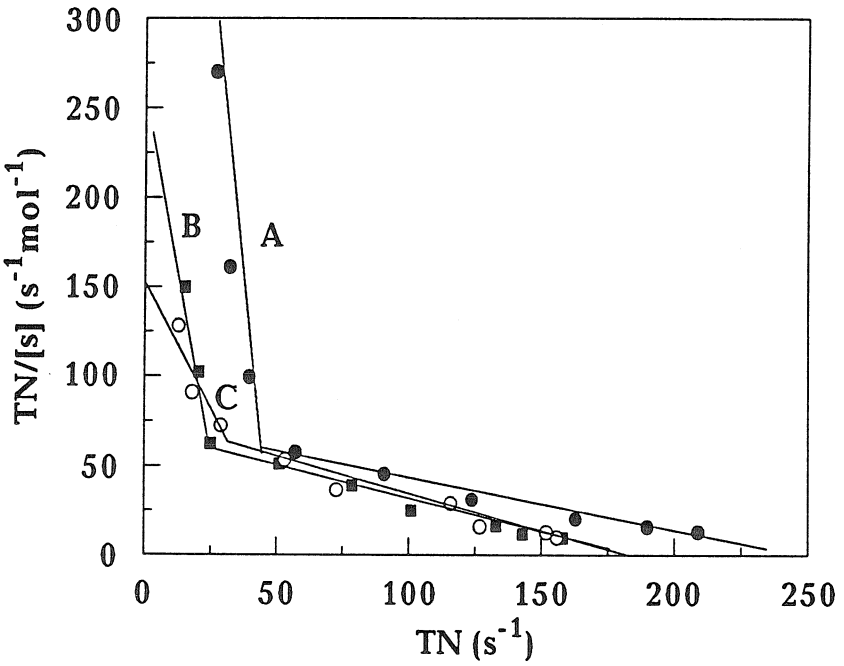
* 5 mM of Tris buffer, 0.1 % Tween-80, pH 7.4 was used. Concentration of TNP-ATP is 30 μM in all experiments.

Table 7. Binding of cytochrome c with native, and 8-azido ATP modified CcO at low[a] and high[b] ionic strength

Sample	Ka (at low salt)[a] (μM^{-1})	Ka (at high salt)[b] (μM^{-1})
native CcO	134.5	0.186
8-azido-ATP modified CcO	9.34	0.107
native CcO with 10 mM of free ATP		0.112
native CcO with 30 μM of TNP-ATP	8.45	0.139

[a] ionic strength is 5 mM
[b] ionic strength is 105 mM

Figure VI.10. Comparison of the effect of TNP-ATP, and ATP on the steady-state kinetics of CcO. The activity assay of CcO in the absent and present of ATP or TNP-ATP was carried out as detailed under experimental. (A) control. (B) in the presence of 3 mM of ATP, (C) in the presence of 30 μM of TNP-ATP.


300
250
200
150
100
50
0
0
50
100
150
200
250
TN/[s] ($s^{-1}mol^{-1}$)
TN (s^{-1})
A
B
C

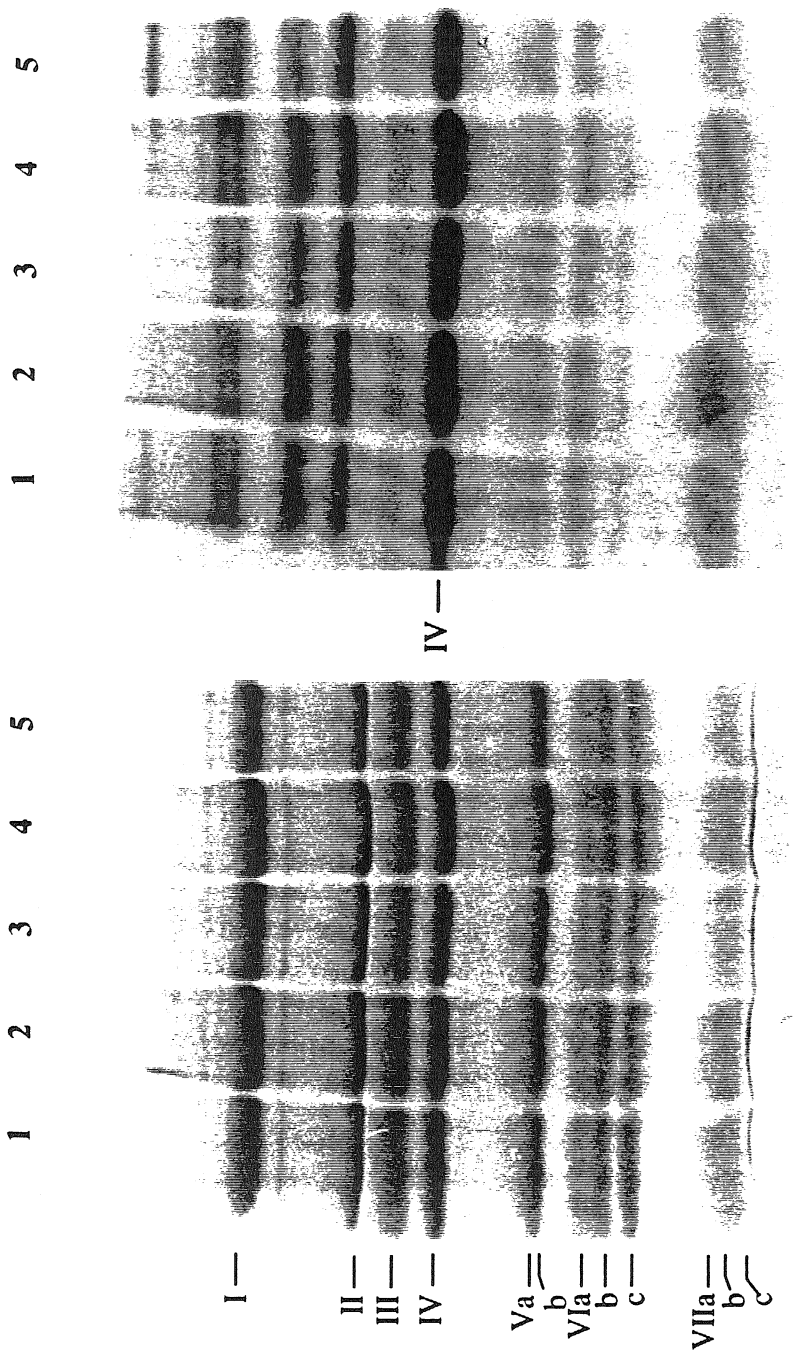
similarly to ATP at a concentration of 3 mM. Both greatly diminish the high affinity phase and decrease the rate of the low affinity phase. The effect of ATP was first reported by Ferguson-Miller *et al.* (1976). These results in the steady-state kinetics provide us will an important link on the relevancy of the TNP-ATP binding to CcO to that of ATP.

Labeling of CcO with 8-azido-[γ-^{32}P] ATP. The results of 8-azido-[γ-^{32}P]ATP-modification of CcO are shown in Figure VI.11. As shown by the autoradiography on the right panel, subunit IV is strongly labeled. Some weak labeling is also observed for subunit III and the nuclear coded subunits. However, no labeling is observed for subunit I and II. The labeling is non-competitive for all the labelled subunits, as ATP or TNP-ATP do not change the labeling intensity. The aspect of the labeling has been discussed before by Huther and Kadenbach (1988b). The labeling on subunit IV was first demonstrated by Montecucco, *et al* (1986) and then by Huther and Kadenbach (1988b), and we conform their experiments.

DISCUSSION:

The principal objective of this study is to determine the binding affinity of TNP-ATP to CcO. Because of its favorable fluorescence properties of TNP-ATP, it may be used as a substitute for ATP, an allosteric effector on the terminal step of the respiratory chain, particularly if it is a more effective effector. Unfortunately, experiments designed to examine the thermodynamics of binding of a small molecule like ATP or TNP-ATP to a membrane protein are often times complicated by paralleled binding of the small molecule to the detergent particle or lipid bilayer of which the protein is also a integral part for biological function. This means that the relevant thermodynamics for the protein in the functional state can only be derived from studies on the protein in detergent or reconstituted into lipid bilayer after allowing for the parallel binding by the detergent or lipid membrane.

Figure VI.11. SDS gel electrophoresis of cytochrome c oxidase photolabeled with 8-azido-[γ-^{32}P]ATP. Right panel, the subunits on the gels are monitored by autoradiography, left panel, the subunits on the gels are discerned by Coomassie Blue stain. The concentration of 8-azido-ATP was 3 μM in all samples. 3 mM ATP (lane 2), 30 μM TNP-ATP (lane 3), 30 μM EDTA (lane 4), 30 μM EGTA (lane 5) may also be added to some samples. Other conditions were specified in experimental.


1 2 3 4 5
1 2 3 4 5
IV
I
II
III
IV
Va
b
VIa
b
c
VIIa
b
c

The partitioning of small amphiphilic molecules into biological membrane, synthetic bilayer vesicles and detergents has been a subject of considerable activity over the years (Conrad and Singer, 1981; Gains and Dawson, 1982; Leonard *et al.*, 1989; Antunes-Madeira and Madeira, 1985; Jones and Lee, 1985). In fact, the partitioning of TNP-ATP to reduced Triton X-100 has ahead been described by Tummino and Gafni (1992). However, to study the interaction of TNP-ATP with isolated CcO, knowledge of the interaction of TNP-ATP with different detergent is necessary. We have, therefore, carried out extensive experiments to determined the interaction of TNP-ATP with different detergents.

Both TNP-ATP and TNP-AMP partition into various detergent micelles (Tween-80, Brij 35, Triton X-100, Lauryl-D-maltoside) with very high affinity. In contrast, ATP itself shows very low affinity toward detergent micelles. The comparison of the detergent behavior of TNP-ATP and ATP, suggests that the TNP group is important in the partitioning. Also, TNP-AMP has a higher binding affinity toward detergent micelles than TNP-ATP ($\Delta(\Delta G)$ = 4-5 KJ/mol). Hydrophobic interaction between TNP group and detergent must therefore provides the driving force for the partitioning. The reason why Reimann and Kadenbach's (1992) failed to observe the saturation of the binding of TNP-ATP to reconstituted CcO could be due to the interfering of the partitioning of TNP-ATP to the lipids, which is difficult to account for in experiments on reconstituted samples.

TNP-ATP binds much stronger to CcO than ATP. The affinity of this binding depends on the detergents used with the protein. From the evaluation of thermodynamic parameters, TNP-ATP very likely binds to CcO by an interaction of triphosphate group with CcO and an interaction of TNP group with detergent. If this model is valid, the free energy of the binding of TNP-ATP to CcO would be determined by two major contributing factors: (1), DG_1, the free energy of TNP group partitioning into the detergent, which should be close to the free energy of TNP-ATP partition in the detergent (about -18 KJ/mol in Tween-80 and -23 KJ/mol in Lauryl-D-maltoside); and (2), DG_2, the free energy of triphosphate group interaction with CcO, which should be close

to the free energy of the ATP binding to CcO (DG = -14 KJ/mol). The summation of these two contribution gives DG = -32 KJ/mol and -37 KJ/mol for the binding of TNP-ATP to CcO in Tween-80 and Lauryl-D-maltoside respectively, which are close to the value we observed in our experiments (DG = -29 KJ/mol and -33 KJ/mol, respectively). The more positive ΔG's from the direct measurement could be simply due to the distortion of the TNP group upon partitioning or triphosphate group binding in the TNP-ATP binding to CcO.

The stoichiometry for TNP-ATP binding to CcO was determined to be 1:1, rather than 2:1 (Reimann and Kadenbach, 1992) as suggested by previous work. This result indicates that there is only one high affinity binding site for TNP-ATP binding to CcO. ATP have been shown to have an effect on the CcO activity when added on both the matrix and cytosolic side of the reconstituted CcO vesicle, albeit with different characteristics (Huther and Kadenbach, 1988). The effect of ATP when added on the cytosolic side has been shown to be purely electrostatic and does not saturate, which is similar for CcO isolated from either bovine heart or *Paracoccus denitrificans*. This interaction is most likely due to an ionic strength effect on the Cc:CcO interaction rather than any specific interaction with CcO. On the other hand, the effect of ATP when added on the matrix side of the bovine CcO, is more specific and shows saturation characteristics (Huther and Kadenbach, 1988). This matrix ATP related interaction is totally absent in the *Paracoccus* CcO. The interaction, thus, very likely involves specific interactions between ATP and one or more nuclear DNA encoded subunit(s) of CcO. If this is the case, the TNP-ATP high affinity binding site in CcO we observe should be related to the ATP binding accessible from the matrix side.

The TNP-ATP high affinity binding to CcO shows a similar effect as 8-azido-ATP-modification of CcO as well as free ATP binding on the binding affinity between cytochrome c and CcO. An 10-fold decrease in the affinity of CcO for cytochrome c has been observed on both TNP-ATP binding as well as 8-azido-ATP-modification under the condition of low ionic strengths. At high ionic strengths, TNP-ATP, ATP and 8-azido-ATP-modification have similar, but more minor effects, on

the binding affinity between CcO and cytochrome c. It is evident that the TNP-ATP binding CcO has resulted in a conformational change in the enzyme that has altered the exposure of those domains near subunit II that are paramount to the docking of cytochrome c. Since the interaction of TNP-ATP with CcO is almost certain to be in the matrix side, the effect on the cytosolic cytochrome c binding site in the subunit II of CcO is allosteric. This conclusion agrees well with the results of earlier studies of Bisson *et al.*, (1987), who showed that the accessibility of those carboxyl amino acids in subunit II, which are important to the cytochrome c binding, was reduced towards the carbodiimide modification (EDC or CMC) in the presence of ATP. Changes in the surface carboxyl's on subunit II will, of course, greatly perturb the binding of cytochrome c and can result in decreasing affinity between the protein redox partners under the condition of low ionic strengths. At physiological ionic strengths, however, the charge interaction between cytochrome c and CcO contributes less to the binding affinity and the binding of ATP, TNP-ATP or 8-azido-ATP-modification should have less significant effects on the binding affinity. Aside from the similarity of the effects on the affinity between cytochrome c and CcO, the TNP-ATP binding to CcO also exhibits similar effect on steady state kinetics of CcO as free ATP. Thus, the high affinity binding of TNP-ATP to CcO we observe is relevant to that of the ATP-interaction.

One interesting aspects of the TNP-ATP binding to CcO is that excessively high concentration of ATP was not able to reverse the TNP-ATP binding. Similar phenomenon has also been observed in the labeling of CcO with 8-azido-[γ-^{32}P]ATP, in which ATP or TNP-ATP were not competed with the cross-linking. The non-competivity of the ATP binding or modification with 8-azido ATP were also observed in the studies of ATP interaction with other membrane proteins. One possible reason for this non-competitive behavior of ATP binding to CcO could be the special properties of this type of ATP binding site, which is very close the lipid bilayer (or may be in the lipid bilayer).

The modification of CcO with 8-azido ATP greatly reduced the apparent binding affinity of TNP-ATP with CcO. It does suggest that the ATP moiety of 8-azido ATP occupied the binding site for

TNP-ATP. Therefore, the 8-azido ATP modification should be able to indicate the subunit on which TNP-ATP bind. 8-azido-[γ-^{32}P]ATP modification of CcO reveals that the subunit IV is the subunit for 8-azido ATP modification and very probably for TNP-ATP binding.

Subunit IV of bovine heart CcO is a polypeptide with 147 amino acid. It consists of a membrane stretch region of about 20 amino acid flanked by hydrophilic domains. It consists of a metal center, Mg^{+2}, (Lin, *et al.*, 1993) associated with the hydrophilic region close to C terminal, of which amino acid sequence is relatively better conserved than the region close to N terminal among different eukaryotes CcO sources. The topology of the subunit IV of bovine heart CcO has been extensively investigated by Malatesta *et al.* (1983). The N terminal region (from amino acid 1-71) of subunit IV protrudes to the matrix side of the membrane and exposes extensively to water. The region of amino acid 72-123 is in contact with lipid bilayer, while the C terminal region from amino acid 127-147 is not accessible to either water or lipid bilayer. Malatesta *et al* (1983) suggested that the N terminal and C terminal face the matrix and cytosolic side of the inner mitochondrial membrane, respectively, although it could not be unambiguous determined that the C terminal face the cytosolic side. The possibility of the c terminal facing the matrix side could not be excluded. One of the putative ligand for Mg^{2+} in subunit IV, Glu 114 (Glu 136 of the precursor) (Lin *et al.*, 1993), is located in the region with high lipid accessibility (amino acid 72-123). If the Mg^{2+} ion provide the binding site for ATP, it, thus, should be close to lipid bilayer. The protection of Mg^{2+} from water soluble reagents could be the reason for the difficulty of removing Mg^{2+} from CcO, and it is obvious that the interaction between TNP-ATP with detergent should be the result of the binding of TNP-ATP to this site. However, more evidence is required to unambiguously determine the role of Mg^{2+} ion in the binding of ATP.

In conclusion, TNP-ATP binds to subunit IV of CcO with high affinity (may be from the matrix side of the CcO) and forms 1:1 complex. The binding affinity of TNP-ATP to CcO is significantly higher than that of ATP. The higher affinity of the TNP-ATP binding are due to the partition of

TNP group into the detergent surrounding this ATP binding site. This high affinity binding of TNP-ATP to CcO mimic the effect of ATP binding on either binding affinity of CcO with cytochrome c or the steady-state kinetics. TNP-ATP, thus, provide us with an alternative tool for investigate the ATP-CcO interaction without significantly change ionic strength of the solution used. The unusual location of this ATP binding site (close to the lipid bilayer, may be even in the lipid bilayer) could be the reason of the non-competivity of the binding.

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