Electron Transfer from Cytochrome *c* to 8-Azido-ATP-Modified Cytochrome *c* Oxidase[†,‡]

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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 flash photolysis 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 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 50% of the intracomplex electron-transfer rate (k_{et}) and 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 nonsaturation 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 either the Cu_A or the cytochrome *a* site were noted, as evidenced by a 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.

Cytochrome *c* oxidase (CcO),[1] the terminal component of the mitochondrial respiratory chain, catalyzes the transfer of electrons from cytochrome *c* to dioxygen and couples electron transfer to the active transport of protons across the mitochondrial inner membrane. The steady-state oxidation of cytochrome *c* catalyzed by this enzyme shows distinctive biphasic behavior (Nicholls, 1964; Ferguson-Miller *et al.*, 1976). The V_{max} values for the high- and low-affinity phases are 10−40 and 100−200 s^{-1}, respectively (Errede & 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 great 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. 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 subunits IV and VII upon photo-cross-linking 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 kinetic effect on the oxidase activity similar to that of ATP (Bisson, *et al.*, 1987).

Several groups have attempted to mimic the specific effects of ATP on the steady-state kinetics of the 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 & Wallace, 1991, 1993). Similar kinetic effects have been observed for 8-azido-ATP-modified CcO as for CcO in the

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[1] The subunit nomenclature of Kadenbach (Kadenbach *et al.*, 1983) is used throughout this paper.

[2] Abbreviations: CcO, cytochrome *c* oxidase; ATP, adenosine 5′-triphosphate; 8-azido-ATP, 8-azido-adenosine 5′-triphosphate; 8-azido-ADP, 8-azido-adenosine 5′-diphosphate; TNP-ATP, 2′ (or 3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; 5-DRF, 5-deazariboflavin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane hydrochloride.

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presence of moderate ATP concentrations (Huther & 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 binding affinity or electron-transfer pathways, or both.

MATERIALS AND METHODS

Materials. 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 ion−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, and 0.1% lauryl D-maltoside. 8-Azido-ATP or 8-azido-ADP (3 mM) 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 1 h. 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 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 and 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 & 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′(3′)-*O*-(2,4,6-Trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP) binds tightly to CcO with a 1:1 stoichiometry ($K_d \sim 3.5$ μM, in 0.1% Tween-80), and this binding is competitive with 8-azido-ATP modification of the enzyme (J. Lin and S. I. 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 TNP-ATP with 10 μM CcO (or 8-azido-ATP-modified CcO) in 5 mM Tris and 0.1% Tween-80 buffer, pH 7.4, in a Centricon-100 microconcentrator at 3300 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 concentrations of TNP-ATP in the filtrate and the original solution, together with that of CcO in the original solution, were determined by UV−visible spectroscopy.

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·A] is the concentration of the enzyme−TNP-ATP complex. C_A^F and C_A^O are the concentrations of TNP-ATP in the filtrate and the 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 cytochrome *c* to native and 8-azido-ATP-modified CcO, a preequilibrated solution of 10 μM cytochrome *c* with 10 μM 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 3300 rpm for 10 min at 3 °C. The microconcentrator retains CcO but allows free cytochrome *c* to pass the membrane. After the centrifugation, the concentrations of cytochrome *c* in the filtrate and the original solution, together with that of CcO in the original solution, were determined by UV−visible spectra. 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* and native CcO, either 10 mM ATP or 30 μM TNP-ATP was also present in some of the low- and high-salt experiments with native CcO.

Steady-State Kinetics of Native and 8-Azido-Modified CcO. The steady-state turnover of CcO was determined by the polarographic method. Cytochrome *c* (0.1−16 μM) was added to 5 nM native or 8-azido-ATP-modified oxidase in 25 mM Tris, 5 mM ascorbate, 0.1% lauryl D-maltoside, and

Table 1: Binding of Cytochrome *c* with Native and 8-azido-ATP-Modified CcO at Low[a] and High[b] Ionic Strength

sample	K_a (at low salt)[a] (μM^{-1})	K_a (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 free ATP		0.112
native CcO with 30 μM TNP-ATP	8.45	0.139

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

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, OH). 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 = moles of cytochrome *c*/second per mole CcO at 20 °C.

Transient Electron Transfer from Cytochrome c to Native CcO with 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 (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 (PBBO (2-(4-biphenylyl)-6-phenylbenzoxazol-1,3)) 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 equilibration for both native CcO and its 8-azido-ATP adduct:

$$\text{ferricyanide} + \text{cytochrome } a^{2+} \Leftrightarrow \text{ferrocyanide} + \text{cytochrome } a^{3+}$$

Solutions of $K_4(Fe(CN)_6)\cdot 3H_2O$ (422 mg) and $K_3(Fe(CN)_6)$ (329 mg) were prepared in 10 mL each of degassed water. CcO (or 8-azido-ATP-modified CcO) was diluted with a solution of 5 mM Tris, 100 mM NaCl, and 0.1% lauryl D-maltoside, pH 7.0, to a final concentration of 30 μM in a total volume of 0.9 mL in a plastic cuvette. In some samples, ATP was added to the buffer to a concentration of 3 mM. The samples then were sealed with rubber stoppers 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] =

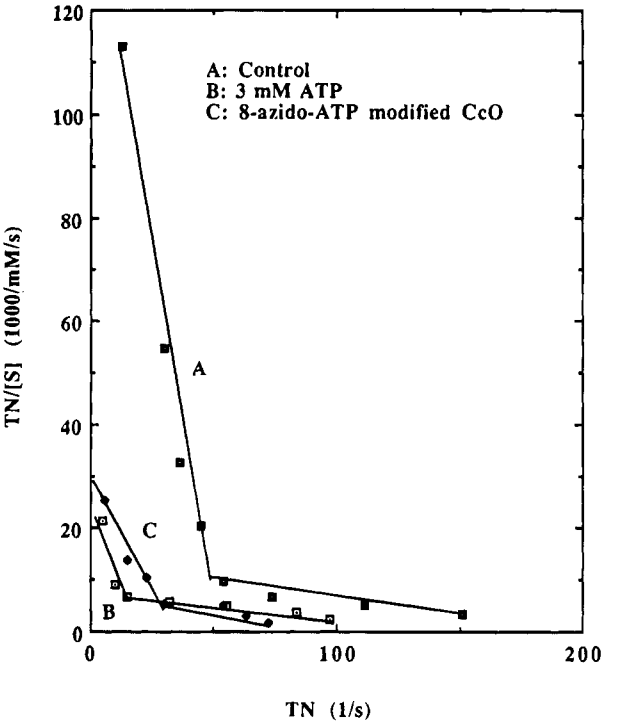

FIGURE 1: Effects of 8-azido-ATP modification and ATP binding on the steady-state kinetics of CcO. The activities of native CcO, native CcO in the presence of 3 mM ATP, and 8-azido-ATP-modified CcO were assayed as detailed in Materials and Methods. (A) Native CcO (control); (B) CcO in the presence of 3 mM ATP; (C) 8-azido-ATP-modified CcO.

1:1:1:1, final concentration of 20 μM; Li *et al.*, 1992) was employed as redox mediator 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 dithionite (5 mg in 100 μL) to the samples. The redox potential of cytochrome *a* was calculated from the following expression:

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

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:

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

First, the CcO (with 20 μM redox mediator described above) was fully reduced by sodium dithionite. Cytochrome *c* (20 μM) 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

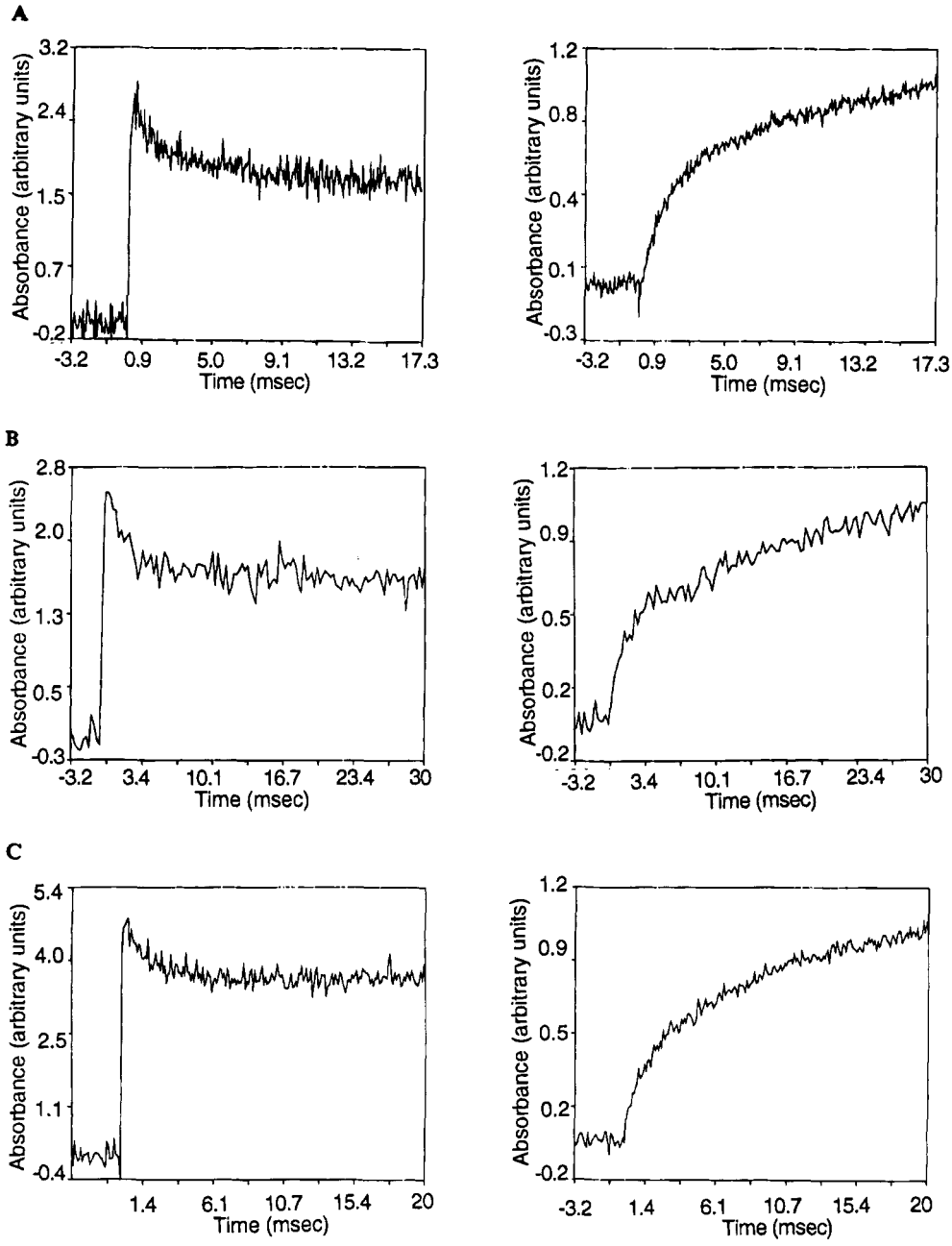

FIGURE 2: Intracomplex electron transfer from ferrocytochrome *c* to native CcO, the CcO−ATP complex, and 8-azido-ATP-modified CcO. Left panels: Reduction of cytochrome *c* by photogenerated flavin semiquinone and reoxidation of ferrocytochrome *c* by various CcOs followed at 550 nm. Right panels: Reduction of cytochrome *a* in the various CcOs 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 CcOs, 10 μM; cytochrome *c* concentration, 10 μM; ionic strength, 105 mM. Other experimental conditions are detailed in Materials and Methods.

potential of Cu_A was determined from the expression

$$E^0_{Cu_A} = 0.265 - 0.059 \log([Cu_A^{+1}]/[Cu_A^{+2}][\text{ferricytochrome } c]/[\text{ferrocytochrome } c])$$

The concentration ratios were determined from the Cu_A^{2+} and cytochrome *c* absorbances at 830 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

Extent of 8-Azido-ATP Modification of CcO. TNP-ATP forms a relatively strong 1:1 complex with CcO ($K_d \sim 3-4$ μM in Tween-80), and this binding is competitive with 8-azido-ATP modification (J. Lin and S. I. Chan, unpublished data). Modification of CcO with 8-azido-ATP reduces the apparent stoichiometry of binding of TNP-ATP to CcO from 0.95 to 0.36. The observed TNP-ATP binding stoichiometry

indicates that 65% of the oxidase molecules were labeled by 8-azido-ATP.

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 ($K_d \sim 0.005$ μM), and this interaction is strongly ionic strength dependent. As shown in Table 1, this interaction is also dramatically reduced (in excess of 10-fold) 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 slightly 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 1 mM (Bisson *et al.*, 1987; J. Lin and S. I. 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 would no longer be in the low ionic strength regime. To complicate matters further, ATP binds directly to cytochrome *c*, also at millimolar concentrations ($K_d \sim 5$ 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, J. Lin and S. I. 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 1, TNP-ATP binding exhibits effects similar to those of 8-azido-ATP modification on the association of cytochrome *c* to CcO at low ionic strength: the interaction is about 10-fold 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 CcOs, 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 CcO and its 8-azido-ATP adduct are represented as Eadie−Hofstee plots 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 labeled, 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 an inhibitory effect on CcO similar to that of 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 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 shows typical transient kinetic traces observed for the intracomplex electron transfer between horse cytochrome *c* and fully oxidized native bovine CcO at a 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 displayed somewhat slower electron transfer kinetics. Figure 2C 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% unlabeled 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 (Figure 2B) and 6 mM ATP. The apparent rate constants for the reoxidation of cytochrome *c* are 412 and 362 s^{-1}, respectively, and the corresponding rate constants for the reduction of cytochrome *a* are 426 and 366 s^{-1}.

As a control, a Cu_A-depleted CcO was also investigated. The kinetic constants deduced from the transients at 550 and 604 nm for a sample for which the Cc/CcO mole ratio is 1:1 are 335 and 347 s^{-1}, respectively (data not shown). A sample of the 8-azido-ADP-modified CcO was also studied. Here, we obtain an apparent rate constant of 796 s^{-1} under otherwise identical conditions. These data are included in Table 2.

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_d 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,

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

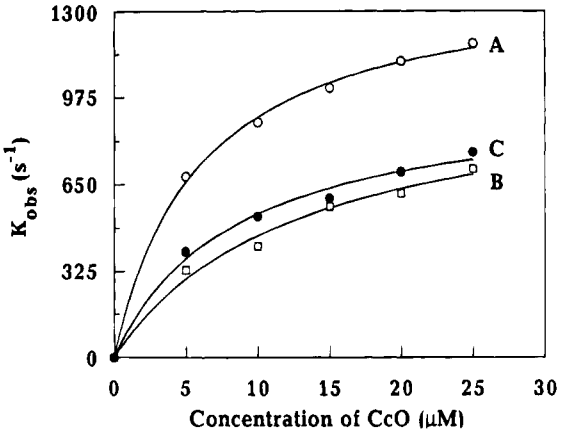

FIGURE 3: Kinetics of electron transfer between ferrocytochrome *c* (10 μM) and various CcOs as a function of CcO concentration (5−25 μM) at an ionic strength of 110 mM. The reaction conditions are described in Materials and Method. The pseudo-first-order rate constants for the reduction of cytochrome *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 eq 3. (A) Native CcO; (B) CcO in the presence of 3 mM ATP; (C) 8-azido-ATP-modified CcO.

Table 2: Observed Rate Constant for Electron Transfer from Cytochrome *c* to Heme *a* of CcO ([Cc]:[CcO] = 1:1)[a]

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 3 mM ATP	419	47
native CcO in the presence of 6 mM ATP	364	41
Cu_A-depleted CcO	341	39

[a] Cc = cytochrome *c*.

Table 3: Kinetic Parameters for Intracomplex Electron Transfer between Cytochrome *c* and Cytochrome *c* Oxidase

sample	K_a [$\times 10^{-4}$ (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)

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

The best fits of the kinetic data obtained for native CcO, 8-azido-ATP-modified CcO, and CcO in the presence of 3 mM ATP are shown in Figure 3, and the results are tabulated in Table 3. We see that either ATP binding or covalent attachment of ATP to the ATP-binding site of CcO decreases 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 that of 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% labeled. In addition, it should be noted that k_{et} for the native CcO (1430 s^{-1}) obtained in this study is lower than that observed in our earlier study (2580 s^{-1}) (Pan *et al.*, 1991). The intracomplex electron transfer rate constant does vary from batch to batch of enzyme; the activity of the oxidase in the batch used in the present study happens to be lower. Our present value of k_{et} is close to that previously reported by Hazzard *et al.* (1470 s^{-1}; Hazzard *et al.*, 1991). We stress, however, that the *effects* of 8-azido-ATP modification of CcO on the electron input reported here are not enzyme batch dependent.

In an effort to obtain more reliable electron input rates for the modified and ATP-bound CcOs, we have corrected for the contribution of the unlabeled or uncomplexed enzyme to the observed transients. On the basis of the existence of two CcO species and assuming that the on−off rates of the cytochrome *c* from the docking site(s) are rapid compared to the intracomplex electron-transfer rates within the two possible types of complexes, the observed rate constant should be given by

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

where K_a and K_a' are the formation constants for the cytochrome *c* complexes with native CcO and 8-azido-ATP-modified (or ATP-bound) CcO, respectively; k_{et} and k_{et}' denote the corresponding intracomplex electron-transfer rates; k_{-1} and k_{-1}' denote the corresponding off-rates of the cytochrome *c* from the respective complexes; and x is the fraction of the sample that is 8-azido-ATP-modified or ATP-complexed.

Using the k_{et} and K_a reported earlier for the native enzyme, the value of k_{et}' and K_a' could be deduced from eq 3 for 8-azido-ATP-modified CcO and native CcO in the presence of 3 mM ATP. Since $K_a \sim K_a'$ under high ionic strengths, we can assume that either (1) $k_1 \sim k_1'$ or (2) $k_{-1} \sim k_{-1}'$, without introducing significant errors. The results of the best fits of the kinetic data obtained for 8-azido-ATP-modified CcO and CcO in the presence of 3 mM ATP, assuming that 65% of the CcO is modified in the 8-azido-ATP-modified CcO sample and 75% of the CcO is bound in the sample containing 3 mM ATP, are tabulated in parentheses in Table 3. Compared to that of native CcO, K_a is reduced by 56% in the case of 8-azido-ATP-modified CcO and 76% for CcO with 3 mM ATP present, and k_{et} is reduced by 50% and 51% for the 8-azido-ATP-modified and ATP-bound enzymes, respectively.

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 ADP have also been determined. These results are summarized in Table 4. 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

Table 4: Redox Potentials of Heme *a* and Cu_A of CcO

sample	redox potential of heme *a* (mV)	reodx potential of Cu_A (mV)
native CcO	321	264
8-azido-ATP-modified CcO	325	275
native CcO in the presence of 3 mM ATP	322	252
native CcO in the presence of 3 mM ADP	317	278

removed from Cu_A in the fully reduced oxidase 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 potential 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. 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, does not perturb the ligand environment of cytochrome *a* or Cu_A significantly. These spectroscopic conclusions augment those derived earlier from the redox potential measurements. Taking these conclusions together, we must further 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.

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 to ATP, we have compared the effects of 8-azido-ATP modification with the effects of ATP binding in the presence of 3 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* is 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 nonspecific ionic strength effects or any indirect interaction between ATP and cytochrome *c* on the docking of cytochrome *c* to CcO. Wallace and co-workers (Craig & 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 & Wallace, 1991), any effects arising from this specific cytochrome *c*−ATP interaction should be minor 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 (J. Lin and S. I. 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 decreases the binding affinity of CcO for cytochrome *c* and slows down the electron transfer from cytochrome *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. ATP binding and 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 must 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 directly into cytochrome *a* (Pan *et al.*, 1991). It appears that both the rates and 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 on 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 (1000−3000 s^{-1}) (Hazzard *et al.*, 1991; Pan *et al.*, 1991, 1993; Larsen *et al.*, 1992). 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 of transfer into the two electron acceptors, namely, Cu_A and cytochrome *a*, and the 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-(dimethylamino)propyl)carbodiimide (EDC) and 1-cyclohexyl-3-(2-morpholinoethyl)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 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 ferrocytochome *c* directly to cytochrome *a* only.

Electron-transfer processes in biological systems are complicated, particuarly 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 electron input pathway and the reduction in the electron-transfer rates from cytochrome *c* to cytochrome *a* could only be due to changes in the electronic coupling between the electron donor and the two potential electron acceptors 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 and 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 (Q.-Z. He, L.-P. Pan, and S. I. Chan, 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 & Malmström, 1986). 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 accomplished 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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