8-Azido-ATP Modification of Cytochrome *c*: Retardation of Its Electron-Transfer Activity to Cytochrome *c* Oxidase[†,‡]

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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 to 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.

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).[1] The effect of ATP on cytochrome *c*−CcO kinetics was first reported by Margoliash and co-workers (Ferguson-Miller et al., 1976). Kadenbach, Bisson, and Montecucco and their co-workers (Huther & 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 electron transfer rates and the efficiency of biological energy transduction. On the other hand, Wallace and co-workers (Corthesy & Wallace, 1986, 1988; Craig & 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 p*K* is on the order of 3−4 mM (Craig & 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-azidoadenosine 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 evidence of deleterious effects on the electron-transfer activities between these redox protein partners. In any case, non-covalent ATP could be competed off of its cytochrome *c* binding site even if the cytochrome *c*:CcO docking involves the same site, provided the docking interaction is sufficiently strong. Such cannot be the case with the covalent ATP adduct.

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

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[1] Abbreviations: CcO, cytochrome *c* oxidase; CcP, cytochrome *c* peroxidase; ATP, adenosine 5′-triphosphate; 8-azido-ATP, 8-azidoadenosine 5′-triphosphate; 8-azido-ADP, 8-azidoadenosine 5′-diphosphate; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; EDTA, ethylenediaminetetraacetic acid; 5-DRF, 5-deazariboflavin; Tris, tris(hydroxymethyl)aminomethane hydrochloride.

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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 noncovalent ATP binding to cytochrome *c*.

MATERIALS AND METHODS

Materials. 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 & Beinert, 1974). The enzyme preparation was stored at −78 °C before use. Enzyme concentrations were determined from the absorbance change ΔA_{red-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 and Wallace (1993). The sample was then loaded onto a CM-cellulose column and eluted with a linear gradient of 5−50 mM phosphate buffer, pH 7.4. Two major peaks (labeled fractions 1 and 2) were obtained and identified by the optical spectral change in the 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 similarly, 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 weights of the native and 8-azido-ATP-modified cytochrome *c*'s were determined by MALDI time-of-flight mass spectrometry on a Vestec Voyager RP BioSpectrometry workstation at the Protein/Peptide Micro Analytical Facility of the 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 a 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 ATP with 10 μM 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 through the membrane, for 20 min at 3 °C. After the centrifugation, the concentrations of ATP in the filtrate and the original solution together with that of cytochrome *c* in the original solution were determined by UV−visible spectroscopy.

To determine the binding contants of the different cytochrome *c*'s to CcO, a preequilibrated solution of 10 μM cytochrome *c* or the ATP(ADP)−cytochrome *c* adduct with 10 μM 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 through the membrane, for 10 min at 3 °C. 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 spectroscopy. Finally, to determine the effect of noncovalent ATP binding to cytochrome *c* on the binding constant between native cytochrome *c* and CcO at low ionic strength, 30 or 300 μM 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

$$[S] = C_S^F$$

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

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

in which [S] is the concentration of free substrate (cytochrome *c* 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 concentrations of substrate in the filtrate and the original solution, respectively; C_E^O is the concentration of enzyme in the original solution. Similar relationships 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. One to 16 μM native or 8-azido-ATP-modified cytochrome *c* was added to 5 nM oxidase in 5 mM Tris, 100 mM KCl, 5 mM ascorbate, 0.1% lauryl-D-maltoside, and 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). The data are presented in Eadie−Hofstee plots. The activity is expressed as the molecular turnover number [TN = (moles of cytochrome *c*) s^{-1} (moles of cytochrome *c* oxidase)$^{-1}$] 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 oxidase in a buffer of 5 mM Tris containing 1 mM EDTA, 0.1% lauryl maltoside, 33 μM

Table 1: Molecular Mass (amu) of Native and 8-Azido-ATP-Modified Cytochrome *c*[a]

sample	molecular mass peak 1 (major)	molecular mass 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 is 503 amu.

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 (PBBO (2-(4-biphenylyl)-6-phenylbenzoxazole-1,3) at 395 nm). The optical signals represented the accumulation of 30 flashes.

Redox Potential of Cytochrome c and the 8-Azido-ATP Adducts. The redox potentials 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 the adduct to the CM-cellulose column is reduced and the adducts are eluted from column ahead of unmodified cytochrome *c*. The ATP−cytochrome *c* adducts (fractions 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 (Table 1). 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 and 12 860 amu of fractions 1 and 2 correspond to single modification by 8-azido-ATP. The minor mass peaks at 12 478 and 12 372 amu from fractions 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 fractions 1 and 2 of the ATP−cytochrome *c* adducts, the two fractions most likely represent only differences in the site of ATP cross-linking, as evidenced by the similarities in the binding of the adducts in the two fractions to CcO as well as their kinetic behaviors. Craig and Wallace (1995) have recently demonstrated by mapping with a trinitrophenyl-containing photoaffinity label that fractions 1 and 2 correspond to ATP adducts with attachment sites at lysine residues 86 and 87, respectively.

Table 2: Apparent Binding Stoichiometry of ATP to Native and 8-Azido-ATP(-ADP)-Modified Cytochrome *c* at Low Ionic Strength (5 mM)

sample	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-ADP-modified cytochrome *c*	0.9

Table 3: Binding Affinity of Native and 8-Azido-ATP(-ADP)-Modified Cytochrome *c* to CcO under Low and High Ionic Strengths

sample	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.

Effect of 8-Azido-ATP(-ADP) Modification of Cytochrome c 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 was determined by the fast filtration method following equilibration of 30 μM ATP with 10 μM 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, although the effect is less significant. This latter result with the ADP adduct confirms the pivotal role of Arg 91 as the point of interaction for the terminal phosphate of ATP in the binding site.

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 (fractions 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* (the K_d between ATP and cytochrome *c* in 10 mM Tris−cacodylate buffer was determined to be 40 μM; Craig & Wallace, 1991) as well as that between cytochrome *c* and CcO at low ionic strengths, we have attempted to

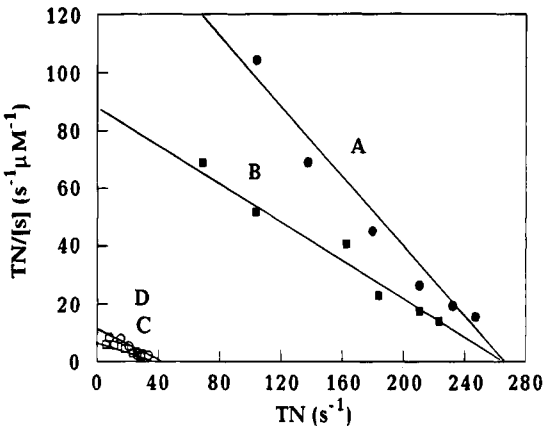

FIGURE 1: Eadie–Hofstee plots of the steady-state kinetics of native and 8-azido-ATP(-ADP)-modified cytochrome *c* with CcO. The activity of CcO in the presence of native and 8-azido-ATP(-ADP)-modified cytochrome *c* was assayed as detailed under Materials and Methods: (A) native cytochrome *c*, (B)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

Table 4: Kinetic Parameters Derived from the Polarographic Assays

sample	K_m (μM)	V_{max} (s^{-1})
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

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* for 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*'s are depicted in Figure 1. Since the concentration range of cytochrome *c* used corresponded 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 cytochrome *c* 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 the data of 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. This smaller effect of ADP adduct formation on the steady-state kinetics of CcO, as well as on the docking interaction between cytochrome *c* and CcO noted earlier, suggests that the effects of nucleotide tethering are not due to lysine modification alone.

Transient Absorption Study of the Electron Input from Native and 8-Azido-ATP-Modified Cytochrome c to CcO. Figure 2, panels A and B, show 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 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 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 941 s^{-1} is obtained for the fast phase. The reduction of cytochrome *a* in the CcO was followed at 604 nm (Figure 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 significantly slower electron-transfer kinetics (see Figure 2C,D). Figure 2C shows the reoxidation of cytochrome *c*, and Figure 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

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

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 eq 1 are shown in Figure 3, and the results are tabulated in Table 5. 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).

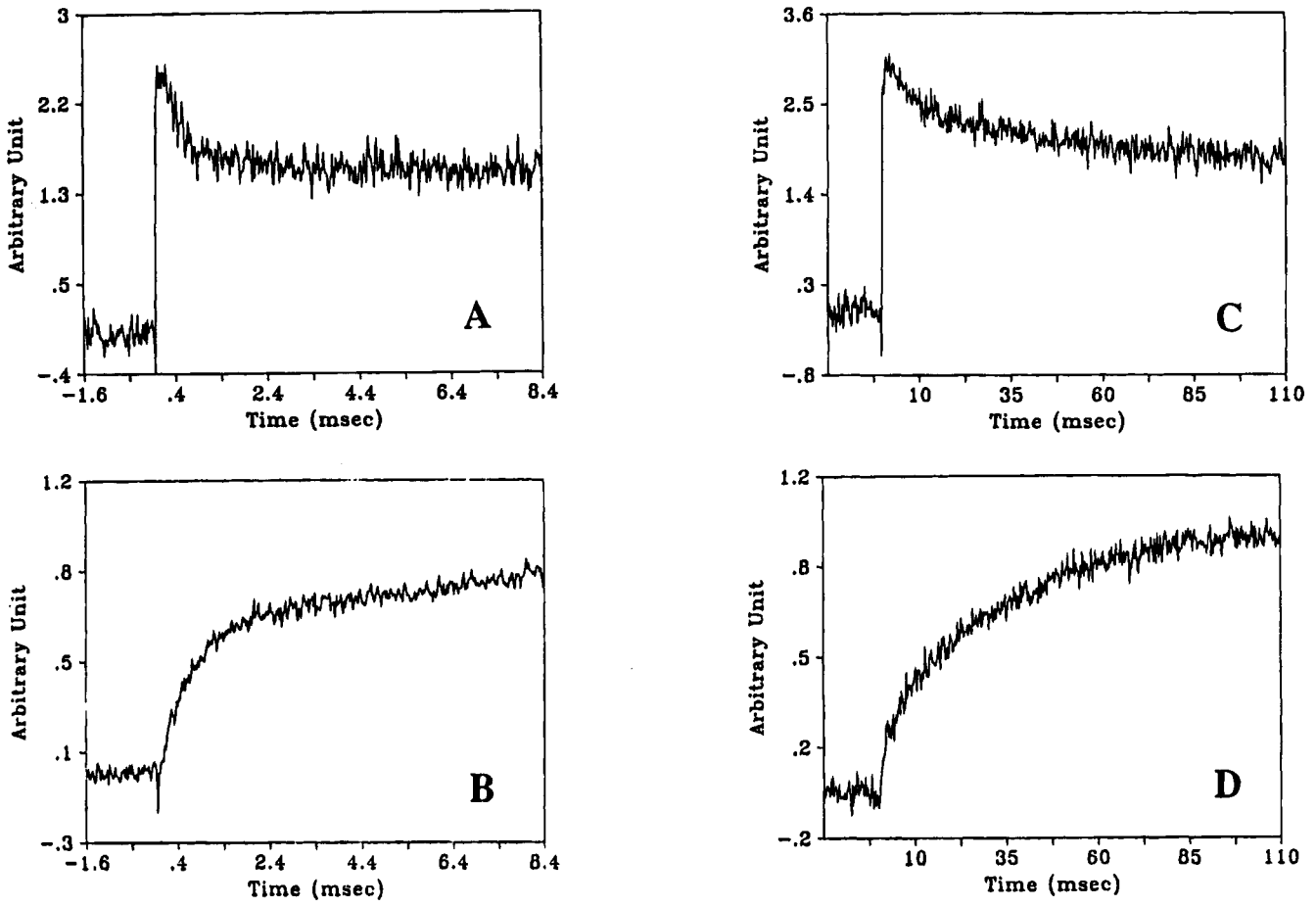

FIGURE 2: Intracomplex electron transfer to native CcO from ferrocytochrome *c* and one of its 8-azido-ATP-modified adducts. (A) Reduction of cytochrome *c* by photogenerated flavin semiquinone and reoxidation of ferrocytochrome *c* followed at 550 nm. (B) Reduction of cytochrome *a* by ferrocytochrome *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 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.

Redox Potentials of Native and 8-Azido-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 $E = E^{0\prime} - 0.059\log$([ferrocyanide]/[ferricyanide]). The value of $E^{0\prime}$ for the ferrocyanide/ferricyanide couple is taken to be +0.43 V.

For the native cytochrome *c* and fractions 1 and 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 been difficult to determine. Peptide mapping of the ATP-labeled amino acid has proved to be infeasible, 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-^{32}P]ATP with cytochrome *c* after cross-linking with a UV lamp in the presence of CcO following manipulation of the sample. Thus, it appears that the adducts between 8-azido-ATP and cytochrome *c* are relatively labile or unstable, and the covalent linkage is readily broken upon manipulation of the adducts. In very recent experiments, however, Craig and Wallace (1995) have successfully mapped the site of cross-linking with a trinitrophenyl-containing photoaffinity label and identified lysine residues 86 and 87 as the site(s) of chemical modification.

Upon formation of the ATP adducts, cytochrome *c* loses some of its ability to bind to CcO. 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 2 relative to the native cytochrome *c* at physiological ionic strength. Under conditions of low ionic strengths, the

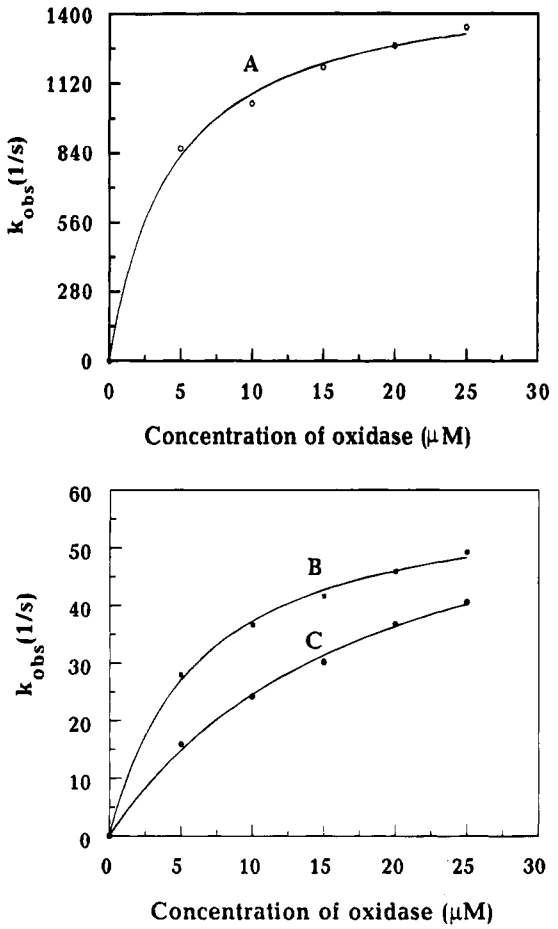

FIGURE 3: Kinetics of electron transfer between ferrocytochrome *c* (10 μM) and CcO at various CcO concentrations (5−25 μM) and an ionic strength of 110 mM. The concentration of cytochrome *c* remained unchanged as the concentration of CcO was varied. The reaction conditions are described in Materials and Methods. 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 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 Figure 2.

Table 5: Kinetic Parameters for Intracomplex ET between Cytochrome *c* and Cytochrome *c* Oxidase

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

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. Support for these arguments comes from crystallographic studies of the structure of cytochrome *c* peroxidase (CcP) and cytochrome *c* complexes (Pelletier & Kraut, 1992), where it has been shown that CcP−Cc(Horse) grown at low ionic strengths exhibits shorter distances between potential charge-mediated hydrogen bond 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 as 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. Of course, the docking of cytochrome *c* to the oxidase could also displace the bound ATP if the docking interaction is sufficiently strong.

It is now generally agreed that Cu_A is the primary electron acceptor in CcO, although an 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 1500−2500 s^{-1} (Pan et al., 1991, 1993; Hazzard et al., 1991; Larsen et al., 1992). The electron transfer rate from Cu_A to heme *a* has been determined to be around 2×10^4 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 the 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 behaviors of the two electron-donating systems under low ionic strength conditions could be the different pathways 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 strength exerts 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 has been studied by Ferguson-Miller et al. (1978) by the chemical modification method. Lysines 8, 13, 72, and 87 have been demonstrated to be important in the docking of cytochrome *c* to CcO. Upon modification of these lysines, dramatic changes in the binding constant and V_{max} have been observed. Osheroff et al. (1980) have also shown that lysine 86 (and to a lesser extent lysines 27 and 73) is also a participant. The docking surface predicted by the above studies has been shown to be almost identical to that for the docking to cytochrome *c* peroxidase as revealed by the recent crystal structure of the CcP−Cc complex (Pelletier & Kraut, 1992). All of the lysines on cytochrome *c* predicted to be important in the binding to CcO are also involved in the docking to CcP to varying extents.

Although we failed to locate the site of 8-azido-ATP modifications by peptide mapping of ATP−cytochrome *c* adduct 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 & Wallace, 1993). In the structure of cytochrome *c*, lysines 86, 87, and 88 are located close to arginine 91, which is the ATP binding site on cytochrome *c*. Thus, one of these lysines could become modified by 8-azido-ATP. In fact, Craig and Wallace (1995) have now shown that either lysine 86 or 87 is modified in the protocol used to form the ATP adducts here.

The modification of cytochrome *c* with 8-azido-ATP, but not the electrostatic binding of free ATP to cytochrome *c*, interferes significantly with the docking of cytochrome *c* to CcO according to our binding studies. It is evident that certain residues (for example, lysine 86 or 87) on cytochrome *c*, which are important to the docking to CcO, have been modified, distinct from electrostatic ATP binding sites. On the basis of this reasoning, we surmise that ATP−cytochrome *c* adducts assume 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.

Any 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 the acceptor regardless of the intervening medium. On the other hand, Gray and coworkers (Beratan et al., 1991; Wuttke et al., 1992) assert that the 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 has been given (Evenson & 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 one or more altered electron-transfer pathways (nonexponential 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-limiting 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 bioenergetics of a cell. A few studies have contributed to our understanding of this feedback control. Most of the studies have focused on the 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 & Kadenbach, 1986, 1987, 1988; Antonini et al., 1988; Reimann & Kadenbach, 1992). However, in a recent study by Craig and Wallace (1993), sensitivity of the respiratory chain to the energetic state of the cell was attributed to the direct binding of ATP to cytochrome *c* and the consequences of this binding to the inhibition of respiration. The efficacy of cytochrome *c*−ATP adducts in restoring dioxygen consumption in cytochrome *c*-depleted mitochondria relative to native cytochrome *c*, reported recently by Craig and Wallace (1993), is 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 mitochondrial membrane (Huther & 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 the binding of cytochrome *c* to CcO or the electron input rate (J. 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 designed 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 a lower binding affinity and a 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 non-covalent 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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