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Modulation of the Cytochrome *c* Oxidase Activity by ATP: Implications for Mitochondrial Respiratory Control

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Abstract: ATP and ADP are potential regulators of mitochondrial respiration and at physiological concentrations they affect the rate of electron transfer between cytochrome *c* and cytochrome *c* oxidase. The electron transfer, however, depends on the electrostatic interaction between the two proteins. In order to exclude any nonspecific ionic effects by these polyvalent nucleotides, we used 2'-O-(2,4,6)trinitro(TNP)-derivatives of ATP and ADP which have three orders of magnitude higher affinity for cytochrome *c* oxidase. A simple titration of the fluorescence intensity of TNP by cytochrome *c* oxidase showed a binding stoichiometry of 2 : 1 cytochrome *c*:cytochrome *c* oxidase. Higher ionic strength was required for TNP-ATP than for TNP-ADP to be dissociated from cytochrome *c* oxidase, indicating that the negative charges on the phosphate group are at least partially responsible for the binding. In both spectrophotometric and polarographic assays, addition of ATP (and ADP to a less extent) showed an enhanced cytochrome *c* oxidase activity. Both electron paramagnetic resonance and fluorescence spectra indicate that there is no significant change in the cytochrome *c*-cytochrome *c* oxidase interaction. Instead, reduction levels of the cytochromes at steady-state suggest that the increased activity of nucleotide-bound cytochrome *c* oxidase is due to faster electron transfer from cytochrome *a* to cytochrome a_3, which is known to be the rate limiting step in the oxygen reduction by cytochrome *c* oxidase.

Key words: ATP, cytochrome *c* oxidase, respiratory control.

A mitochondrion as the major energy producing site in an aerobic cell is expected to somehow sense the energization state of the cell and control mitochondrial respiration. As the respiration is coupled to proton pumping across the inner mitochondrial membrane, the chemiosmotic theory (Mitchell and Moyle, 1965) predicts that the proton electrochemical potential gradient ($\Delta\mu_H^+$) is an important modulator of the respiration. Among the respiratory chain complexes in the inner mitochondrial membrane, cytochrome *c* oxidase has been proposed to be the regulation site. The crystal structure (Iwata *et al.*, 1995; Tsukihara *et al.*, 1995) and catalytic mechanism (Han *et al.*, 1990; Babcock and Wikström, 1992) of the enzyme are now known. The enzyme receives electrons from cytochrome *c* and reduces molecular oxygen which is bound to cytochrome a_3. The electron transfer within the cytochrome *c*-cytochrome *c* oxidase complex follows the pathway: cytochrome *c*→cytochrome *a* (Cu_A)→cytochrome a_3 (Cu_B)→O_2. Two components of $\Delta\mu_H^+$, namely the membrane potential ($\Delta\psi$) and the pH gradient (ΔpH), are known to independently control different steps in the electron transfer pathway (Gregory and Ferguson-Miller, 1989; Nicholls and Butko, 1993). $\Delta\psi$ modulates the rate of electron transfer from cytochrome *c* to cytochrome *a* whereas ΔpH affects electron transfer from cytochrome *a* to cytochrome a_3.

Intracellular concentration of ATP, or the ratio [ATP]/[ADP], is another measure of energization state of the cell. Low level of ATP should stimulate the respiratory chain to obtain energy for phosphorylating ADP. Therefore it is a possibility that these nucleotides may well regulate the respiration rate by modulating the enzymic activity of the respiratory chain complexes. The effect of ATP on the cytochrome *c* oxidase activity was indeed reported some twenty years ago (Ferguson-Miller *et al.*, 1976). When measured polarographically, the rate of electron transfer decreased by millimolar ATP at low concentrations of cytochrome *c*. Many other workers (Hüther and Kadenbach, 1986; Monteccuco *et al.*, 1986; Bisson *et al.*, 1987; Malatesta *et al.*, 1987) have also reported similar results that can be summarized as: (i) ATP abolishes the low K_m phase of the biphasic kinetics of cytochrome *c* oxidation by cytochrome *c* oxidase; (ii) V_{max} of the reaction also increases

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in the presence of ATP.

The site of ATP binding, however, remains controversial. Wallace and coworkers (Craig and Wallace, 1991, 1995) argued that ATP binds directly to cytochrome *c* affecting its affinity towards cytochrome *c* oxidase and/or efficiency of the intramolecular electron transfer within the cytochrome *c*-cytochrome *c* oxidase complex. Using a photoaffinity labeling technique in conjunction with site-directed mutagenesis, they proposed that ATP binds to cytochrome *c* near conserved arginine-91. Lin *et al.* (1995) found that the binding affinity is reduced by an order of 3. They proposed that a change in the docking conformation of the cytochrome *c* to cytochrome *c* oxidase is responsible for the retarded electron transfer. The model has been criticized, however, by the fact that the affinity for cytochrome *c* is in the order phosphate≫ATP~ADP but the inhibitory effect of ATP is much higher than that of phosphate. This argues that ATP regulates the rate of cytochrome *c* oxidation by binding to cytochrome *c* oxidase rather than binding to cytochrome *c*.

Photoaffinity labeling techniques have been useful in locating the binding sites of ATP in cytochrome *c* oxidase. Earlier work suggested subunits IV and VIII to be the binding sites (Bisson *et al.*, 1987). Binding of ATP is postulated to cause conformational changes in cytochrome *c* oxidase which in turn lowers the affinity of cytochrome *c* oxidase towards cytochrome *c*. Hüther and Kadenbach (1986, 1987) analyzed the kinetics of reconstituted cytochrome *c* oxidase and suggested that there are two binding sites, one each on the cytosolic and matrix side. A major progress in this respect has been made recently by Taanman *et al.* (1994) who utilized a yeast strain which lacks the gene for subunit VI. By comparing the stoichiometry of ATP binding and the activities of the wild type and mutant, they concluded that there are two binding sites in cytochrome *c* oxidase: one on subunit VIa deactivates the enzyme and the other at an unknown location activates the enzyme. They suggested the former to be the regulating site for cytosolic ATP.

Electron transfer between cytochrome *c* and cytochrome *c* oxidase occurs through the formation of cytochrome *c*-cytochrome *c* oxidase complex. Interaction between these two redox partners is electrostatic in nature. Therefore ions, especially polyvalent anions, have a large influence on the electron transfer kinetics (Kossekova *et al.*, 1989; Cooper, 1990). Since ATP is a tetravalent anion and K_d of ATP-cytochrome *c* oxidase complex is estimated to be in the range of mM, it will have a non-specific electrostatic effect in addition to a specific allosteric effect, if any (Hüther and Kadenbach, 1986). In order to observe the allosteric effect without interference of nonspecific charge effect, one can covalently attach ATP to cytochrome *c* oxidase by photoaffinity labeling. However, reactive photoproducts sometimes attack nearby protein moeity which is remote from the binding site. Moreover, in the native state, ATP never forms a covalent bond with cytochrome *c* oxidase. A better alternative is to increase the affinity of ATP towards cytochrome *c* oxidase through chemical modification of ATP. TNP-ATP, a fluorescent analog of ATP, was found to have much higher affinity (K_d in the range of μM; see Taanman *et al.*, 1994) and therefore modulates the cytochrome *c* oxidase activity at micromolar concentrations. At such a low concentration of TNP-ATP, the nonspecific electrostatic effect can be ignored.

With the advantages conferred by TNP-ATP, we reexamined the effects of ATP on the electron transfer between cytochrome *c* and cytochrome *c* oxidase. The reaction rates were measured both spectrophotometrically and polarographically on detergent-solubilized and reconstituted enzyme. Reduction levels of the cytochromes in the cytochrome *c*-cytochrome *c* oxidase complex were also determined to estimate the effects of ATP on the individual electron transfer steps within the complex. Binding of TNP-nucleotides were also characterized in detail. Influence of ATP binding on the cytochrome *c*-cytochrome *c* oxidase interaction was examined by both electron paramagnetic resonance (EPR) and fluorescence spectroscopy.

Materials and Methods

Materials

Cytochrome *c* from horse heart (type VI), yeast cytochrome *c*, asolectin (type IIS), HEPES, ATP, and ADP were purchased from Sigma Chemical Co. (St. Louis, USA) and used without further purification. 2′-O-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP) and TNP-ADP were from Molecular Probes (Eugene, USA). (1-Oxyl-2,2′,5,5′-tetramethylpyrroline-3-methyl)-methanethiosulfonate (MTSSL), a thiol-specific spin-label, was obtained from Reanal (Budapest, Hungary). All other chemicals were of highest grade available.

Preparation of ferrocytochrome *c*

Cytochrome *c* was reduced by excess ascorbate in the presence of small amount of TMPD, an electron mediator, and the unreacted chemicals were removed by a subsequent gel filtration on a Sephadex G-15 column. When operated anaerobically, more than 95% reduction was easily achieved. Concentration was measured using $\varepsilon_{550}=27\ mM^{-1}\cdot cm^{-1}$.

Spin-labeling of yeast cytochrome *c*

Thirty mg of yeast cytochrome *c* was dissolved in 5 ml of 10 mM potassium phosphate, pH 7.0 and treated with a 2-fold molar excess of dithiothreitol to dissociate any disulfide bridged dimers. The solution was incubated for 1 h at room temperature under nitrogen and then excess dithiothreitol was removed by a small Sephadex G-15 column. A 1.5-fold molar excess of MTSSL in ethanol (final ethanol concentration <1%) was added to cytochrome *c* and the mixture was incubated for 2 h at room temperature to label the cysteine residue at position 102. Unreacted spin-label was removed by gel filtration on a Sephadex G-15 column.

Purification and reconstitution of cytochrome *c* oxidase

Cytochrome *c* oxidase was isolated from beef hearts according to Yonetani method (Yonetani, 1961). Concentration was determined using $\varepsilon_{605-630}$(red-ox)=27 $mM^{-1} \cdot cm^{-1}$. The enzyme was reconstituted into asolectin vesicles by a cholate dialysis method (Casey *et al.*, 1982). Usually more than 80% of cytochrome *c* oxidase was inserted with right-side-out orientation.

Kinetic measurements

Electron transfer rates were measured both polarographically and spectrophotometrically. In a typical polarographic assay, 0.6 μM cytochrome *c* oxidase was mixed in an appropriate buffer with 2 mM cytochrome *c* and the reaction was initiated by adding 25 mM ascorbate and 0.2 mM TMPD. When the oxygen dissolved in the medium is exhausted by the electrons supplied by ascorbate/TMPD *via* cytochrome *c*, absorbance at 550 nm arising from ferrocytochrome c suddenly increases. Rates were estimated from the time it takes for the oxygen to be exhausted. During the steady-state, where absorbance at 550 nm stays constant, absorption spectrum was obtained and compared to the spectrum of fully reduced cytochrome *c* and cytochrome *c* oxidase to measure the reduction level of cytochrome *c* (550 nm) and cytochrome *a* (605 nm). In a spectrophotometric assay, 5 nM of cytochrome *c* oxidase or cytochrome *c* oxidase vesicles was mixed with 40 μM ferrocytochrome *c* and the absorption at 550 nm was monitored to follow the oxidation of cytochrome *c*. The decay curve was fit by a first-order reaction and the rate constant (and turnover number) was calculated accordingly. In case of cytochrome c oxidase vesicles, the rates were measured for both coupled state and uncoupled state, which were generated in the absence and in the presence of uncouplers (valinomycin and CCCP), respectively. Spectra were collected on an SLM-Aminco DW2000 UV-Visible spectrophotometer.

Other spectroscopic measurements

Fluorescence spectrum of TNP-derivatives of ATP and ADP in the presence and absence of cytochrome *c* oxidase were measured by exciting at 410 nm on an SLM-Aminco AB-2 luminescence spectrophotometer. Background emission of the buffer was subtracted from the spectra for a quantitative analysis. EPR spectra of spin-labeled cytochrome *c* which is free or bound to cytochrome *c* oxidase were obtained at room temperature with a flat quartz cell on a Bruker ER-200 X-band spectrometer. Spectral conditions: microwave frequency 9.76 GHz, attenuation 5 dB, modulation 100 kHz, total scan width 100 G.

Results and Discussion

Oxidation of cytochrome *c* by cytochrome *c* oxidase exhibits a biphasic kinetic behavior which is attributed to two binding sites on cytochrome *c* oxidase with different affinity for cytochrome *c* (Ferguson-Miller *et al.*, 1976). A major consequence of ATP binding is abolition of the high affinity (low K_m) phase and at the same time increase in K_m of the low affinity site, as first reported by Ferguson-Miller *et al.* (1976). ATP and ADP bind to cytochrome *c* oxidase with a dissociation constant $K_d \sim 10^{-3}$ M, which is in the range of physiological ATP concentration. Therefore ATP may function as a regulator for mitochondrial respiration under physiological conditions.

Since cytochrome *c* interacts with cytochrome *c* oxidase electrostatically, the electron transfer between cytochrome *c* and cytochrome *c* oxidase depends heavily on ionic strength of the medium (Kossekova *et al.*, 1989). Therefore ATP at millimolar concentrations may well have nonspecific ionic effects (Hüther and Kadenbach, 1986). In order to selectively observe the specific allosteric effects of ATP, one can covalently attach ATP to cytochrome *c* oxidase by photoaffinity labeling. This type of modification, however, may severely alter the properties of the enzyme. Recently Taanman *et al.* (1994) found that fluorescent analogs, TNP-ATP and TNP-ADP, bind to cytochrome *c* oxidase with much higher affinity ($K_d \sim 10^{-6}$ M) and affect the cytochrome *c* oxidase activity in a way similar to native nucleotides but at much lower concentrations. The binding is not covalent so that these analogs can be used as an alternative to the photolabeled nucleotides without problems of nonspecific ionic effects. We further characterized the binding of TNP-nucleotides to cytochrome *c* oxidase and the their effects on individual steps of electron transfer within the cytochrome *c*-cytochrome *c* oxi-

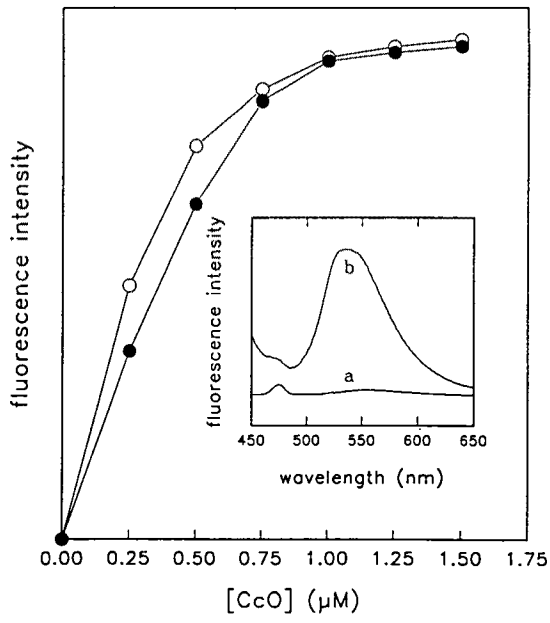

Fig. 1. Changes in the emission intensity due to binding of TNP-ATP and TNP-ADP to cytochrome *c* oxidase. Aliquots of cytochrome *c* oxidase (CcO) were added to 3 μM TNP-ATP in 10 mM K-HEPES (pH 7.5) containing 0.005% (w/v) dodecyl maltoside and the fluorescence intensity at 534 nm was plotted as a function of TNP-ATP (●) and TNP-ADP (○) concentration. Titration was performed in a 1 cm×1 cm quartz cell. The samples were excited at 408 nm and the emission spectra were measured between 450 and 650 nm. In the inset, the fluorescence spectrum of 3 μM TNP-ATP bound to 1.5 μM cytochrome *c* oxidase (b) is compared to the pure TNP-ATP spectrum (a).

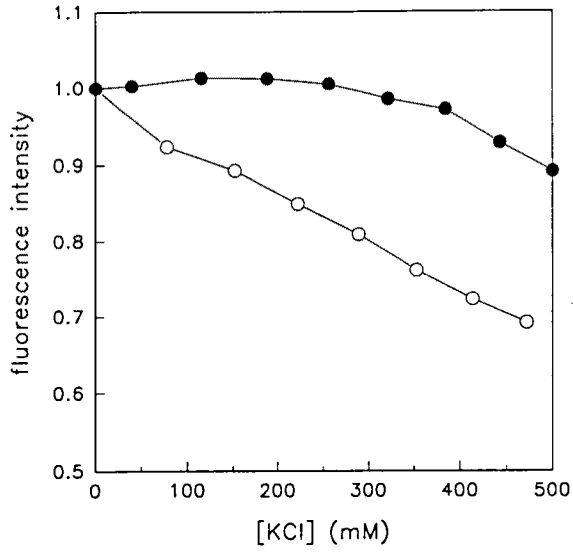

Fig. 2. Dissociation of bound TNP-nucleotides by addition of KCl. 3 μM of TNP-ATP (●) or TNP-ADP (○) was added to 1.5 mM of cytochrome *c* oxidase and the fluorescence intensity at 534 nm was set to 1.0. Relative fluorescence intensity was plotted as a function of added [KCl]. Excitation wavelength was 408 nm.

dase complex.

Characterization of TNP-nucleotides binding

When excited at 408 nm, TNP-ATP fluoresces at ~554 nm (see trace (a) of the inset of Fig. 1). Addition of cytochrome *c* oxidase to TNP-ATP resulted in a tremendous enhancement of fluorescence intensity with a concomitant blue-shift in the wavelength of the emission maximum (from 554 nm to 530 nm), as shown in trace (b) of the inset. This suggests that the environment of the fluorophore became more hydrophobic upon binding to cytochrome *c* oxidase. When 3 μM TNP-ATP was titrated with cytochrome *c* oxidase, the fluorescence enhancement saturates at the cytochrome *c* oxidase concentration of ~1.5 μM (Fig. 1). This simple titration experiment points to the existence of two binding sites for ATP per cytochrome *c* oxidase monomer. Monteccuco *et al.* (1986) first proposed the binding site to be on the subunit IV and VIII based on a photolabeling experiment using 8-azidoATP. Using a mutant cytochrome *c* oxidase lacking subunit VIa, Taanman *et al.* (1994), however, clearly demonstrated that one of the ATP binding site is on the subunit VIa, which is not included in the proposal of Monteccuco *et al.* (1986). After carrying out a complicated analysis of the fluorescence data assuming that the mutant has only one binding site, they concluded that the native cytochrome *c* oxidase has two binding sites for ATP. Our simple titration experiment reached the same conclusion. Similar experiments were performed on vesicular cytochrome *c* oxidase. We attempted to compare fluorescence enhancement by cytochrome *c* oxidase vesicles with that by solubilized cytochrome *c* oxidase to determine sidedness and stoichiometry of nucleotide binding. Strong scattered light by vesicles, however, hampered the measurements. Therefore sidedness and the location of the other binding site are not clear at present.

The binding of TNP-ADP is very similar to that of TNP-ATP as shown in Fig. 1. Since the binding stoichiometry and the binding constant are nearly the same for the two nucleotides, it is very likely that they bind to the same sites on the enzyme. Hüther and Kadenbach (1987) argued that there is a binding site for ADP in the matrix side. Taanman *et al.* (1994) suggested that the binding site on subunit VIa is on the cytosolic side and it is an inhibitory site sensing ATP pool in the cytosol. Therefore it is tempting to postulate that the other site may be on the matrix side, as Hüther and Kadenbach (1987) suggested, sensing ATP pool in the matrix. In this way cytochrome *c* oxidase can monitor ATP/ADP level, which is a measure of energi-

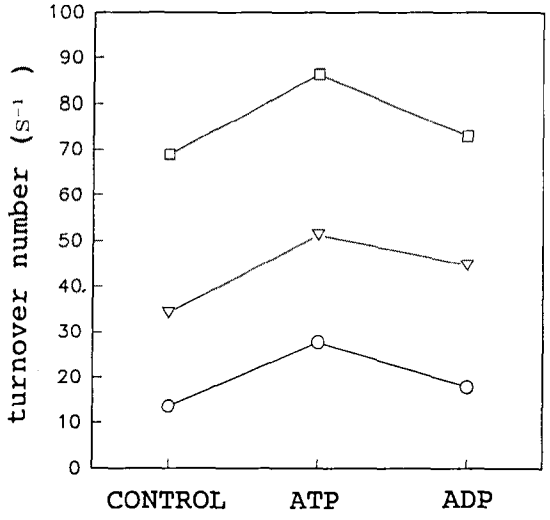

Fig. 3. Effects of ATP and ADP on the cytochrome *c* oxidase activity measured by a spectrophotometric method. To 5 nM cytochrome c oxidase in 10 mM K-HEPES, pH 7.5, and 0.005% dodecyl maltoside was added 40 μM ferrocytochrome *c* and its oxidation was monitored by the absorption at 550 nm. The decay curve was fit by an exponential function to obtain a pseudo first-order rate constant, from which the turnover number (number of electrons transferred per second per cytochrome *c* oxidase molecule) was calculated. Concentration of KCl was 0 mM (○), 50 mM (▽), and 100 mM (□).

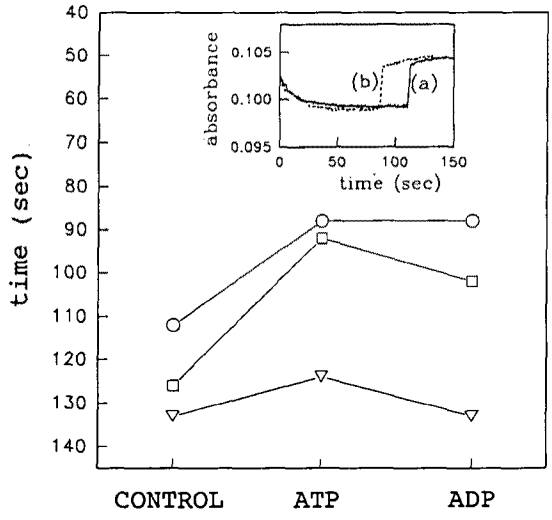

Fig. 4. Effects of ATP and ADP on the cytochrome *c* oxidase activity measured by a polarographic method. 0.6 μM cytochrome *c* oxidase and 2 μM cytochrome *c* were mixed in 10 mM K-HEPES (pH 7.5) containing 0.005% dodecyl maltoside. The reaction of oxygen reduction was initiated by adding 0.2 mM TMPD and 12 mM ascorbate. Time it takes for the reaction system to exhaust dissolved oxygen in the absence (inset a) or in the presence (insert b) of ATP was taken as a measure of reaction rate. Concentration of KCl was 0 mM (○), 50 mM (▽), and 100 mM (□).

zation state of the cell, and control the mitochondrial respiration.

Although the binding characteristics were similar for both nucleotides, response to a high ionic strength was very different. As depicted in Fig. 2, bound TNP-ATP does not readily dissociate from cytochrome c oxidase at KCl concentrations up to ~200 mM. Even at 500 mM, only ~10% of the bound ATP becomes dissociated. On the other hand, the fraction of bound TNP-ADP decreases steadily as [KCl] increases. The difference in the dissociability upon KCl addition can be rationalized by the difference in the charge: ATP is of higher valence than ADP. Therefore electrostatic interaction is at least partially reponsible for the complex formation between the nucleotides and cytochrome *c* oxidase. Hydrophobic interaction is also operative as evidenced by the increased binding affinity of TNP-labeled nucleotides compared to the native nucleotides.

Effects of ATP and ADP on the activity of detergent-solubilized and reconstituted cytochrome *c* oxidase

ATP abolishes the high affinity (low K_m) phase of the kinetics of the oxidation of cytochrome *c* by cytochrome c oxidase. It also increases V_{max} of the reaction. In other words ATP acts as an inhibitor of cytochrome *c* oxidase at low concentrations of cytochrome *c*. Effect of TNP-ATP and TNP-ADP on the cytochrome *c* oxidase activity was measured spectrophotometrically as shown in Fig. 3. In a typical spectrophotometric assay, 5 nM cytochrome c oxidase was mixed with 40 mM ferrocytochrome *c* and absorption at 550 nm was monitored to follow oxidation of ferrocytochrome *c*. The decay curve was fit by a single exponential and the first-order rate constant was calculated accordingly. With such a large excess of cytochrome *c*, the rate becomes V_{max} which can be expressed as a turnover number, i.e., number of electrons transferred per second per cytochrome *c* oxidase molecule. Under the present conditions where large excess ferrocytochrome *c* reacts with cytochrome *c* oxidase, cytochrome *c* after transferring electron to cytochrome *c* oxidase has to be dissociated from cytochrome *c* oxidase before a second molecule of ferrocytochrome *c* binds to cytochrome c oxidase. This means that, if dissociation of ferricytochrome *c* is a rate limiting step, high salt concentration helps cytochrome *c* dissociate from cytochrome *c* oxidase and hence increases the electron transfer rate. This is seen in Fig. 3 where the turnover number increases as ionic strength increases. ATP further enhances the activity to a large extent even at high ionic strength, whereas ADP has smaller effects. Since the concentration of TNP-ATP and TNP-ADP was only 20 μM, one can ignore any nonspecific electrostatic effects of polyvalent nucleotides. Therefore the difference ob-

served is solely due to specific binding of nucleotides which act as effectors.

Electron transfer rates were also measured under polarographic conditions (Fig. 4). Electrons are provided by ascorbate *via* TMPD, a redox mediator, to cytochrome *c* which stays bound to cytochrome *c* oxidase during the catalytic cycle. In contrast to a spectrophotometric assay, formation of the complex is rate limiting in the polarographic assay. Therefore salt at high concentration retards the electron transfer between cytochrome *c* and cytochrome *c* oxidase, which is just opposite to what was observed in a spectrophotometric assay. In the inset of Fig. 4 was shown time trace of absorbance at 550 nm. When all the dissolved oxygen was reduced to water making the reaction medium anaerobic, cytochrome *c* becomes reduced giving a sharp rise in the absorption at 550 nm. The time it takes to exhaust oxygen was taken as a measure of the electron transfer rate. Again, both ATP and ADP enhanced the rate. At high ionic strength ATP was more effective than ADP but at low ionic strength both were approximately equally potent. Vesicular cytochrome *c* oxidase exhibited similar trends but the enhancement of activity by ATP and ADP was small (data not shown).

Effects of ATP and ADP on the individual electron transfer steps

Electron transfer from cytochrome *c* to oxygen, the terminal substrate, follows the path: cytochrome *c*→cytochrome *a* (Cu_A)→cytochrome a_3 (Cu_B)→O_2. Absorption at 550 nm (see inset of Fig. 4) is mostly contributed by ferrocytochrome c whereas 80% of the absorption at 605 nm is due to ferrocytochrome *a*. In order to see which step was affected by the nucleotides, we measured the reduction level of cytochrome *c* and cytochrome *a* at the steady-state. In the absence of the nucleotides without added KCl, 90% and 80% of cytochrome *c* and cytochrome *a*, respectively, are in the ferrous state. Both cytochrome *c* and cytochrome a are more oxidized at 50 mM KCl. Since the rate of oxygen reduction is slower at 50 mM KCl (see Fig. 4), the lower reduction level of the cytochromes shown in Fig. 5 simply indicates that reduction of cytochrome *c* by TMPD is retarded: slower input of electrons from TMPD keeps cytochrome *c* and cytochrome *a* more oxidized if the rate of electron transfer from cytochrome *a* to cytochrome a_3 is not significantly altered. In the past, reduction of cytochrome *c* by TMPD is not considered as a rate limiting process (Gregory and Ferguson-Miller, 1989; Crinson and Nicholls, 1993). Recently, however, Ortega-Lopez and Robinson (1995) pointed out that the electron transfer from TMPD to cytochrome *c* is indeed an important factor that affects

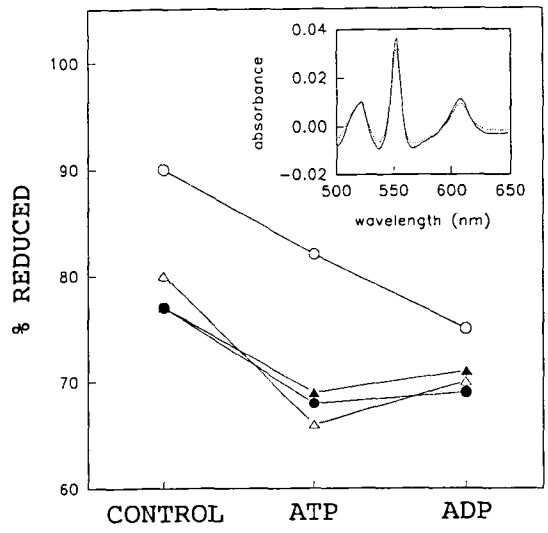

Fig. 5. Reduction level of cytochrome *c* and cytochrome *a* at the steady-state. During the measurements described in Fig. 4, there was a period of time with constant absorbance at 550 nm before the reaction system exhausted dissolved oxygen. A steady-state spectrum was obtained (dotted line in the inset) during the period. Soon after all the dissolved oxygen was reduced to water, all the metal centers in the cytochrome *c*-cytochrome *c* oxidase complex became fully reduced (solid line of the inset). The ratios of absorbance at 550 nm and 605 nm were taken as the reduction level of cytochrome *c* and cytochrome *a*, respectively. Same measurements were made in the presence of nucleotides and at higher KCl concentration. Hollow symbols for the data at [KCl]=0 mM and filled symbols for the data at [KCl]=50 mM. Circles for cytochrome *c* and triangles for cytochrome *a*.

reduction level of both cytochromes. Since the electron transfer TMPD→cytochrome *c* is sensitive to ionic strength of the medium, the data must be interpreted with caution. Nucleotides at such low concentrations, however, are not expected to affect the electron transfer from TMPD to cytochrome *c* so that the observed changes in the reduction levels of cytochromes are due to binding of nucleotides to the enzyme. ATP decreased the reduction level of both cytochrome *c* (from 90 to 82%) and cytochrome *a* (from 80 to 65%). Since the bound ATP increases the rate of oxygen reduction at cytochrome a_3, the above result strongly suggests that the electron transfer from cytochrome *a* to cytochrome a_3 becomes faster in the presence of ATP. In the presence of ADP, reduction of cytochrome *c* decreases a little further but that of cytochrome *a* becomes slightly higher than ATP. Considering that the rate of oxygen reduction is similar for both cases (see Fig. 3), it can be concluded that ADP when compared to ATP makes the electron transfer from cytochrome *c* to cytochrome *a* a little faster but that from cytochrome *c* to cyt a_3 a little slower with keeping electron transfer from cytochrome a_3 to oxygen unaltered. At higher

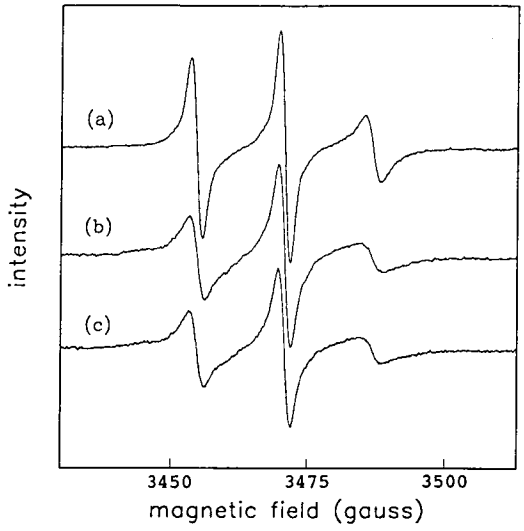

Fig. 6. EPR spectra of spin-labeled cytochrome *c*. Yeast cytochrome *c* (20 μM) was spin-labeled at cysteine-102 and the spectrum was measured in the buffer (a), after addition of equimolar cytochrome *c* oxidase in the absence (b) and in the presence (c) of 40 μM TNP-ATP. Spectra were obtained in a flat quartz cell at room temperature. Spectral conditions: microwave frequency 9.76 GHz, attenuation 5 dB, modulation 100 kHz, total scan width 100 G.

ionic strength, the effects of nucleotides are less salient although tendencies are similar. Since there are two binding sites for ATP in cytochrome *c* oxidase, it is difficult to discriminate the stimulation site from inhibition site (Taanman *et al.*, 1994) and the enhanced activity we observed may be a result of combined effects. Same is true for the ADP binding. As ADP is expected to have opposite effects to ATP, similar trends observed for both ATP and ADP are intriguing. One possibility is that the enhanced V_{max} by ADP is an artifact and the enzyme *in vivo* senses only ATP. The problem can be solved if one can estimate effects of one nucleotide in the presence of the other and at the same time discern a stimulation site from an inhibition site. Many workers (Bisson *et al.*, 1987; Antonini *et al.*, 1988; Reimann *et al.*, 1988) proposed that ATP binds to cytochrome *c* oxidase to induce conformational changes which in turn affects its interaction with cytochrome *c*. Others suggest that ATP binds to cytochrome *c* to weaken the cytochrome *c*-cytochrome *c* oxidase interaction. In order to see if there is indeed a conformational change when cytochrome *c* oxidase binds ATP, we examined the cytochrome *c*-cytochrome *c* oxidase interaction with an EPR spin-label technique. Yeast cytochrome *c* has a unique cysteine residue at position 102 that can be easily labeled by a thiol-specific spin-label. Fig. 6a is the EPR spectrum of spin-labeled cytochrome *c* in the buffer without cytochrome *c* oxidase. The spectral shape indicates that the spin label tumbles with a much slower rotational motion compared to a free spin-label in the buffer, whose spectrum comprises a three lines of equal intensity not shown). When a stoichiometric amount of cytochrome *c* oxidase was added (Fig. 6b) to form a 1 : 1 cytochrome *c*-cytochrome *c* oxidase complex, there is a large change in the spectral shape reflecting significant change in the mobility of the spin label. We added 20 mM TNP-ATP or TNP-ADP to the cytochrome *c*-cytochrome *c* oxidase complex and EPR spectrum was obtained as shown in Fig. 6c. Neither ATP nor ADP caused a significant change in either the shape or the intensity of the spectrum. We also measured resonance energy transfer from AEDANS attached to cytochrome *c* oxidase to the heme of cytochrome *c* (Hall *et al.*, 1988) to find no change in the efficiency of energy transfer upon addition of the nucleotides (data not shown). Since these measurements are proved to be very sensitive to cytochrome *c*-cytochrome *c* oxidase interaction, it can be concluded from the results that binding of the nucleotides to cytochrome *c* oxidase does not cause either dissociation of cytochrome *c* from the enzyme or a major change in the docking conformation between the two proteins.

Conclusion

Using TNP-derivatives of ATP and ADP, we were able to examine the pure effects of these nucleotides on the electron transfer without nonspecific ionic effect. A simple titration of the fluorescence intensity of TNP by cytochrome *c* oxidase showed the binding stoichiometry of TNP-nucleotide:cytochrome *c* oxidase to be 2 : 1. Higher ionic strength was required for TNP-ATP than for TNP-ADP to dissociate from cytochrome *c* oxidase, indicating that the negative charges on the phosphate group are at least partially responsible for the binding. In both spectrophotometric and polarographic assays, addition of ATP (and ADP to a less extent) showed an enhanced cytochrome *c* oxidase activity. Both electron paramagnetic resonance and fluorescence spectra indicate that there is no significant change in the cytochrome *c*-cytochrome *c* oxidase interaction. Instead, reduction levels of the cytochromes at the steady-state suggest that the increased activity of nucleotide-bound cytochrome *c* oxidase is due to faster electron transfer from cytochrome *a* to cytochrome a_3, which is known to be the rate limiting step in the oxygen reduction by cytochrome *c* oxidase.

Acknowledgement

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