ATP-induced spectral changes in cytochrome *c* oxidase

A kinetic investigation

Giovanni ANTONINI,* Francesco MALATESTA,* Paolo SARTI,† Beatrice VALLONE† and Maurizio BRUNORI††

*Department of Experimental Medicine and Biochemical Sciences, University of Rome 'Tor Vergata', Rome, and †Department of Biochemical Sciences and C.N.R. Center of Molecular Biology, University of Rome 'La Sapienza', Rome, Italy

Mixing ATP with soluble oxidized cytochrome *c* oxidase induces a spectral perturbation in the Soret region of the enzyme. This spectral perturbation is observed at ATP concentrations similar to those found to modulate the catalytic activity of cytochrome *c* oxidase [Malatesta, Antonini, Sarti & Brunori (1987) *Biochem. J.* **248**, 161–165]. The process is reversible and corresponds to a simple binding with $K_d = 0.2$ mM at 25 °C. The absorbance change follows a first-order time course, and analysis of the ATP-concentration-dependence indicates the presence of a rate-limiting monomolecular step that governs the process. From the temperature-dependence of this process, studied at saturating concentrations of ATP, an activation energy of 44 kJ/mol (10.6 kcal/mol) was measured. The spectral perturbation also occurs when cytochrome *c* oxidase is reconstituted into artificial phospholipid vesicles, with equilibria and kinetics similar to those observed with the soluble enzyme. Mixing ATP with soluble oxidized cyanide-bound cytochrome *c* oxidase induces a different spectral perturbation, and the apparent affinity of ATP for the enzyme is substantially increased. There is no absolute specificity for ATP, because EGTA, inositol hexakisphosphate, sulphate and phosphate are all able to induce an identical spectral perturbation with the same kinetics, although the value of the apparent K_d is different for the various anions. The presence of Mg^{2+} ions decreases, in a saturation-dependent fashion, the apparent affinity of cytochrome *c* oxidase for ATP. The absorbance change can be correlated to the displacement of the Ca^{2+} bound to cytochrome *c* oxidase.

INTRODUCTION

Cytochrome *c* oxidase (EC 1.9.3.1), the terminal enzyme of the mitochondrial respiratory chain, oxidizes 4 molecules of ferrocytochrome *c*, reducing O_2 to water (Wikstrom *et al.*, 1981). The enzyme, an integral membrane protein, was shown to pump protons from the matrix to the intermembrane space of mitochondria, proton translocation being coupled to electron transfer (Wikstrom, 1977). Both these reactions contribute, in mitochondria, to the build-up of the electrochemical potential gradient that drives the F_0F_1 -ATPase-catalysed synthesis of ATP (Mitchell, 1961).

The enzyme is composed of 12 subunits in mammals (Buse, 1984); the three largest polypeptides are encoded by the mitochondrial genome and are crucial to electron-transfer and proton-pumping activities. Several pieces of indirect evidence support the idea that the nuclear-encoded subunits are involved in the regulation of enzyme activity or its assembly (Kadenbach, 1986).

Different conformational states of cytochrome *c* oxidase have been invoked to account for the control of either electron-transfer or proton-pumping activities (for reviews see Brunori *et al.*, 1987*a,b*). Thus a more active state of the enzyme, called pulsed cytochrome *c* oxidase, has been spectroscopically and kinetically characterized (Antonini *et al.*, 1977). More recently, kinetic evidence for a conformational control of the proton-pumping

activity of the enzyme has led to the suggestion of a linkage between the electrochemical gradient built up across the membrane during catalysis and the functional properties of cytochrome *c* oxidase reconstituted into artificial phospholipid vesicles (Brunori *et al.*, 1985).

Several lines of evidence indicate an ATP-induced allosteric conformational change, that can modulate electron transfer. These can be summarized as follows: (i) ATP at millimolar concentration modifies the pre-steady-state and steady-state kinetics of cytochrome *c* oxidase, either solubilized by detergents or reconstituted into artificial phospholipid vesicles (Ferguson-Miller *et al.*, 1976; Kadenbach, 1986; Bisson *et al.*, 1987; Malatesta *et al.*, 1987); (ii) ATP binds to subunits VIII and IV of cytochrome *c* oxidase in experiments performed with the photoactivable azido-ATP (Montecucco *et al.*, 1986); (iii) ATP induces a change in the water-exposed acidic residues of cytochrome *c* oxidase, as indicated by reaction with water-soluble carbodi-imides (Bisson *et al.*, 1987).

Of course, the fact that in the cell ATP is in the millimolar concentration range suggests some significance for a physiological role of ATP binding to cytochrome *c* oxidase leading to functional modulation of the enzyme activity.

In the present paper we report evidence that ATP and other polyanions alter the optical spectra of soluble cytochrome *c* oxidase and of the enzyme reconstituted

Abbreviations used: $InsP_6$, inositol hexakisphosphate; COV, cytochrome *c* oxidase-containing vesicles.

† To whom correspondence should be sent at the following address: Dipartimento di Scienze Biochimiche, Università 'La Sapienza', Piazzale Aldo Moro 5, 00185 Roma, Italy.

into phospholipid vesicles. Absorbance changes follow first-order kinetics, suggesting that the process is rate-limited by a monomolecular process. Complexing of ATP with Mg^{2+} ions further suggests a role for ATP in the control of cytochrome *c* oxidase activity, possibly mediated by Ca^{2+} deprivation of cytochrome *c* oxidase.

MATERIALS AND METHODS

Enzymes and chemicals

Cytochrome *c* oxidase was prepared as described by Yonetani (1961), but the final suspending buffer contained 0.2% sodium cholate. Cytochrome *c* oxidase concentrations given in the text are expressed in terms of total haem, and thus oxidase concentrations in functional units are calculated by dividing the total haem concentration by 2. Enzyme was stored at -80°C for no longer than 6 months and thawed immediately before use. Cytochrome *c* (type VI), catalase, ATP (type I) and InsP_6 were from Sigma Chemical Co. All other reagents were of analytical grade.

Preparation of phospholipid vesicles

Cytochrome *c* oxidase-containing vesicles (COV) were prepared by the method of Casey *et al.* (1983), with the use of L- α -phosphatidylcholine (type IIS from Sigma Chemical Co.), and were tested for both respiratory-control ratio (> 5) and orientation (see Sarti *et al.*, 1983). The cytochrome *c* oxidase molecules with cytochrome *a* facing the external medium were found to account for 90% of the total, both in the presence and in the absence of InsP_6 and ATP. The last dialysis of COV was against 10 mM-Hepes/KOH buffer, pH 7.5, containing 39.6 mM-KCl and 40.9 mM-sucrose.

Pulsed cytochrome *c* oxidase

The pulsed enzyme was obtained by anaerobically reducing cytochrome *c* oxidase with a minute amount of solid $\text{Na}_2\text{S}_2\text{O}_4$ (0.2 mg/ml) in the presence and in the absence of catalase (10 $\mu\text{g}/\text{ml}$). The reduced enzyme was then exposed to O_2 for a few minutes and the pulsing ratio was determined from the enhanced rate of oxidation of reduced cytochrome *c*, in air, relative to the resting enzyme.

Cyanide-bound cytochrome *c* oxidase

The cyanide complex of the enzyme was obtained by dialysing overnight at 4°C soluble oxidized cytochrome *c* oxidase against 5 mM-Hepes containing 0.5% Tween 80 and 7.5 mM-KCN (pH adjusted to 7.5 with dil. HCl).

Binding experiments

The experiments were carried out with a Cary 219 spectrophotometer or with a Gibson-Durrum stopped-flow apparatus equipped with a 1.5 cm-light-path observation chamber. The slit aperture employed in the stopped-flow experiments (0.5 mm, > 2 nm bandwidth) led to a decrease of the absorbance change recovery to about 75–80% of the expected signal, as shown by an internal calibration carried out at 550 nm using the reaction between ferricytochrome *c* and ascorbate. Therefore static and kinetic difference spectra were superimposed at one wavelength (e.g. 440 nm in Fig. 1), and comparison holds for shape.

Typically, soluble cytochrome *c* oxidase was diluted to

Fig. 1. Difference spectra obtained by addition of anions to soluble oxidized cytochrome *c* oxidase

The continuous line refers to the difference spectrum obtained with 10 μM soluble oxidized cytochrome *c* oxidase in 5 mM-Hepes buffer, pH 7.5, containing 0.5% Tween 80, in a 20°C thermostatically controlled spectrophotometer, after addition of 0.2 mM- InsP_6 to the sample cell and of equivalent volume of buffer to the reference cell. The symbols refer to maximal absorbance changes observed by stopped-flow spectroscopy (between 3 ms and 1 min) on mixing 20 μM -cytochrome *c* oxidase (in 5 mM-Hepes buffer, pH 7.5, containing 0.5% Tween 80) with 1 mM- InsP_6 (*), 4 mM-ATP ($\blacktriangle$), 50 mM- K_2SO_4 ($\blacksquare$) and 200 mM- P_i (potassium salt) ($\blacksquare$) dissolved in the same buffer. The inset shows a kinetic trace at 440 nm. For other details see the Materials and methods section.

20 μM with 5 mM-Hepes/KOH buffer, pH 7.5, containing 0.5% Tween 80, and then dialysed extensively against the same buffer. Before the experiments the enzyme was centrifuged at 14000 *g* for 15 min to remove heavier aggregates.

Stock solutions (100 mM) of InsP_6 and ATP were prepared in water just before the experiments, and adjusted to pH 7.0 to minimize hydrolysis.

RESULTS

On mixing oxidized soluble cytochrome *c* oxidase with different anions (i.e. ATP, EGTA, InsP_6 , sulphate, P_i) a transient was observed in the Soret region. The time course of the process followed a first-order rate law under all the conditions explored. The observed first-order rate constant of the process was wavelength-independent (results not shown), and the amplitude of the absorbance change in the range 400–500 nm yielded a kinetic difference spectrum. In Fig. 1 the static difference spectrum is compared with the kinetic difference spectra obtained in the stopped-flow apparatus on mixing 20 μM -cytochrome *c* oxidase with different anions. Within experimental error the two are taken to be the same. Absorbance recovery at 440 nm indicates that no significant part of the process was lost in the dead-time of the stopped-flow apparatus (3 ms). Moreover, when saturating concentrations of anion were employed the amplitude of the spectral perturbation was the same regardless of the anion used.

Fig. 2. Effect of ATP on soluble oxidized cytochrome *c* oxidase

(a) Maximal absorbance changes observed at 440 nm (between 3 ms and 1 min) as a function of ATP concentration. The points were obtained in the stopped-flow apparatus on mixing 20 μ M-cytochrome *c* oxidase (in 5 mM-Hepes buffer, pH 7.5, containing 0.5% Tween 80) with different concentrations of ATP in the same buffer. ATP concentrations are after mixing. Measurements were made at 25 °C. (b) Observed first-order rate constant for the transient at 440 nm as a function of ATP concentration. All conditions were as indicated for panel (a).

Table 1. Equilibrium parameters observed on mixing oxidized cytochrome *c* oxidase with different anions

Anion	Apparent K_d (mM)	$k_{obs.}$ (s^{-1}) (at saturating concentrations)	Negative charges at pH 7.5
EGTA	<0.03	0.09	4
InsP ₆	0.05	0.11	9*
ATP	0.2	0.10	3.5†
Sulphate	2.5	0.10	2
P _i	12.5	0.12	1.8

* Martin & Evans (1986).

† Alberty (1969).

The reversibility of the process was demonstrated as follows. Cytochrome *c* oxidase (50 μ M) was incubated at room temperature for 10 min with saturating amounts of InsP₆ (1 mM) or ATP (10 mM). Thereafter the solution was either dialysed against buffer or passed through a Sephadex G-25 column equilibrated with the same buffer.

Fig. 3. Arrhenius plot of the rate constant observed upon mixing ATP with cytochrome *c* oxidase

The first-order rate constant observed at 440 nm was measured at different temperatures from 5.5 to 40.0 °C. Cytochrome *c* oxidase (20 μ M) in 5 mM-Hepes buffer, pH 7.5, containing 0.5% Tween 80 was mixed in a thermostatically controlled stopped-flow apparatus with 10 mM-ATP dissolved in the same buffer.

On mixing this preparation of the enzyme with either InsP₆ or ATP the same spectral change obtained with the untreated enzyme was observed.

Ultracentrifuge experiments indicated that the enzyme in the absence and in the presence of 1 mM-InsP₆ was for the largest part (85%) in the dimeric form (sedimentation coefficient values of the order of 12–13 S; see Georgevich *et al.*, 1983), with small amounts of monomers and heavier aggregates.

Two relevant parameters for the apparent binding of ATP to cytochrome *c* oxidase are depicted in Fig. 2. Fig. 2(a) reports the amplitude of the absorbance change observed at 440 nm in the stopped-flow apparatus as a function of ATP concentration; an apparent dissociation constant $K_d = 0.2$ mM was calculated (the continuous line corresponding to the theoretical binding curve). Fig. 2(b) depicts the dependence of the first-order rate constant on ATP concentration. The time course of the process always followed first-order kinetics under all conditions, but it is clear from Fig. 2 that the observed first-order rate constant decreases with an increase in the concentration of ATP, reaching a plateau ($k_{obs.} = 0.1$ s⁻¹) at ATP concentrations greater than 0.5 mM. The rate of the process and the apparent K_d were the same on mixing ATP with the enzyme in the resting or the pulsed state, and addition of catalase (10 μ g/ml) had no effect in either case.

Similar results were obtained with different anions; Table 1 reports a compilation of the relevant parameters. The apparent K_d values for the various anions may be correlated with the affinity of each anion for Ca²⁺. The apparent affinity for cytochrome *c* oxidase is therefore maximal for EGTA and minimal for P_i.

In sharp contrast with these differences in apparent equilibrium constant, the rate of the process and its concentration-dependence were very similar for all the

Fig. 4. Effect of ATP on oxidized cytochrome *c* oxidase reconstituted into artificial phospholipid vesicles

The experiments were performed in the stopped-flow apparatus, mixing 10 μ M-cytochrome oxidase with ATP; ■ refers to soluble cytochrome *c* oxidase, and □ refers to cytochrome *c* oxidase-containing vesicles (COV). The solvent was 10 mM-Hepes buffer, pH 7.5, containing 39.6 mM-KCl and 40.9 mM-sucrose (and 0.5% Tween 80 in the case of soluble cytochrome *c* oxidase); ATP was in the same buffer. Measurements were made at 25 °C.

anions used, including ATP. The first-order rate constant reached in all cases a value of $k_{\text{obs.}} = 0.1 \text{ s}^{-1}$ at saturating concentrations, irrespective of the anion used. This shows that the mechanism underlying complex-formation, as monitored by the spectral perturbation, is independent of the type of anion, and its rate is independent of anion concentration.

Fig. 3 reports the temperature-dependence (shown in the form of an Arrhenius plot) of the observed rate constant for the process observed upon mixing saturating amounts of ATP with cytochrome *c* oxidase. The data afford the calculation of $E_a = 44 \text{ kJ/mol}$ (10.6 kcal/mol) and $\Delta S^\ddagger = 21 \text{ J} \cdot \text{deg}^{-1} \cdot \text{mol}^{-1}$ (5 cal $\cdot \text{deg}^{-1} \cdot \text{mol}^{-1}$).

On mixing dithionite-reduced cytochrome *c* oxidase with an anaerobic solution of ATP, a small (although reproducible) optical perturbation in the Soret region was observed (maximal change at 455 nm), and it was found to be very similar to that reported for reduced enzyme in mitochondria after addition of EGTA (Wikstrom & Saari, 1975).

The apparent binding of ATP to the enzyme reconstituted into small phospholipid vesicles (COV) is shown in Fig. 4, where data obtained with soluble enzyme at the same concentration are reported for comparison. The amplitude of the spectral perturbation is smaller with COV than with the soluble enzyme, but the apparent K_d and the observed first-order rate constant were similar for the two preparations ($k_{\text{obs.}} = 0.1 \text{ s}^{-1}$ at saturating concentration of ATP, with similar concentration-dependence).

On mixing ATP with the cyanide-bound cytochrome *c* oxidase in detergent the kinetic difference spectrum of the observed process is significantly different from that seen with the control enzyme (inset to Fig. 5). It is, however, of significance that with the cyanide-inhibited enzyme the apparent K_d for ATP is smaller than that observed with the control enzyme ($K_d = 0.085 \text{ mM}$) (Fig. 5).

Fig. 5. Effect of ATP on cyanide-bound cytochrome *c* oxidase

The experiments were performed in the stopped-flow apparatus mixing 20 μ M cyanide-bound cytochrome *c* oxidase with ATP. The enzyme was dissolved in 5 mM-Hepes buffer, pH 7.5, containing 7.5 mM-KCN and 0.5% Tween 80; ATP was in the same buffer. Measurements were made at 25 °C. The inset shows the kinetic difference spectrum (■) obtained on mixing 20 μ M cyanide-bound cytochrome *c* oxidase with saturating ATP. * represents the kinetic difference spectrum obtained with control enzyme.

Fig. 6. Effect of Mg^{2+} ions on the apparent binding of ATP to soluble oxidized cytochrome *c* oxidase

The apparent K_d for the binding of ATP to the enzyme is plotted as a function of the $\text{Mg}^{2+}/\text{ATP}$ ratio. Mg^{2+} was added to the ATP-containing syringes as MgCl_2 . All other conditions were as indicated in Fig. 2 legend.

Moreover, the first-order rate constant observed at 450 nm (wavelength of the maximal absorbance change for the cyanide derivative) again reaches a value of about 0.1 s^{-1} at saturating ATP concentrations, increasing when ATP concentration is decreased.

The effect of Mg^{2+} ions on the binding of ATP to native soluble cytochrome *c* oxidase was also investigated in order to assess the role of this metal ion, which under physiological conditions is complexed by ATP. Increasing the $\text{Mg}^{2+}/\text{ATP}$ ratio from 0 to 10:1 leads to a decrease of the apparent affinity of cytochrome *c* oxidase for the anion (Fig. 6). Thus at a $\text{Mg}^{2+}/\text{ATP}$ molar ratio of 4:1 the apparent K_d can be increased approximately 6-fold ($K_d = 1.3 \text{ mM}$); however, a further increase in

Mg²⁺ concentration does not change the value, indicating a saturation behaviour.

The observed first-order rate constant for the process is again independent of the presence of Mg²⁺.

DISCUSSION

The experiments reported above clearly demonstrate the effect of ATP and other anions on the spectrum of oxidized cytochrome *c* oxidase, and document the kinetics of the process.

Kadenbach *et al.* (1988) have reported static spectral changes upon addition of ATP to oxidized cytochrome *c* oxidase (solubilized by lauryl maltoside). The difference spectrum reported by these authors is different from that shown in Fig. 1; it should be noticed, however, that the anion concentration range explored by these authors was much higher (0–70 mM) than that investigated in the present work. In fact with some enzyme preparations we have confirmed that at very high ATP concentrations an optical change different from that reported in Fig. 1 is observed, and therefore it is likely that more than one process may be present.

Under our conditions apparent complex-formation with different anions, as revealed by the spectral change in the Soret region, follows a simple hyperbolic behaviour. All these anions have in common the property of being able to chelate Ca²⁺. Therefore, also in view of the results reported above on the reduced enzyme, we postulate that the observed spectral change and the kinetics of the process may be correlated to the removal of Ca²⁺ bound to cytochrome *c* oxidase.

The kinetics of the process shows that a monomolecular event, possibly coupled to a conformational change of cytochrome *c* oxidase, governs binding. Thus (i) the time course of the reactions is first-order under all conditions (e.g. ATP concentrations from 0.1 to 10 mM), (ii) the same behaviour was observed with ATP, EGTA, InsP₆, P_i and sulphate, (iii) the rate of the process, which is concentration-independent at high anion concentrations, increases with the decrease in the fractional saturation, and (iv) the limiting value of the observed rate constant ($k_{\text{obs.}} = 0.1 \text{ s}^{-1}$) is smaller than typical rates for the binding of small charged molecules to proteins, and for chelation of free Ca²⁺ by specific chelators.

A possible formal mechanism to account for the results demands the presence of a pre-equilibrium between two species of cytochrome *c* oxidase, according to the following scheme:

where A is the prevailing species in the absence of anion (X) and B is the Ca²⁺-free species of the enzyme [which

we assume binds ATP on the basis of the experiments by Montecucco *et al.* (1986) and Bisson *et al.* (1987)]. If the monomolecular interconversion of A into B is slow ($k_{+1} = 0.1 \text{ s}^{-1}$) compared with the second process (k_{+2} in the scheme), the overall process will be limited by the monomolecular conformational change.

The continuous line in Fig. 2(b) is a computer simulation of the dependence of the observed rate constant on ATP concentration, assuming that reaction 2 is always at equilibrium. Under this regime the observed rate constant is expressed by the following:

$$k_{\text{obs.}} = \frac{k_{+1} + k_{-1}}{K_2 \cdot [\text{ATP}]} + k_{+1} \quad \text{with } K_2 = \frac{k_{+2}}{k_{-2}}$$

It may be noticed that if the A → B transition corresponds to the dissociation of bound Ca²⁺ forced by chelation of free Ca²⁺ by X then the same formal mechanism applies.

Description of the data is possible with different sets of values of k_{-1} and K_2 (see Table 2); given $K_d = 0.2 \text{ mM}$ and plausible values for k_{+2} and k_{-2} , a range of values for k_{-1} (0.1 – 10 s^{-1}) and K_2 (14×10^3 – $7 \times 10^5 \text{ M}^{-1}$) is compatible with the results. Finally, at this stage it cannot be decided if the observed optical change is induced by formation of the complex with X or by removal of bound Ca²⁺.

The finding that both resting cytochrome *c* oxidase and pulsed cytochrome *c* oxidase display identical effects upon mixing with ATP (with the same equilibrium and kinetic constants) shows that the process is not modified in the transition from the resting state to the pulsed state. It is possible that different conformational states of cytochrome *c* oxidase may all be present in solution, yielding further support to the contention of multiple states that are differently populated depending on solution components and/or the history of the enzyme (Antonini *et al.*, 1985).

Reconstitution into liposomes has no effect on the rate and affinity of the observed process, although the optical change in the Soret region is clearly diminished. These results suggest the following. (i) The binding site for Ca²⁺ is on the cytoplasmic side of cytochrome oxidase, in agreement with previous results obtained with reduced cytochrome *c* oxidase in mitochondria (Wikstrom & Saari, 1975). It may be noted that both subunits IV and VIII, which bind ATP in photolabelling experiments (Bisson *et al.*, 1987), have one transmembrane segment, and thus also could provide a binding site for ATP on the cytoplasmic surface. (ii) Reconstitution of the enzyme in the phospholipid bilayer has no effect on the rate of the process. (iii) The smaller absorbance change seen may reflect partial chelation of Ca²⁺ by phospholipids and/or steric hindrance to the site.

The observed optical change (Fig. 1) reflects a perturbation in the environment of the cytochromes, although it is difficult to decide if only one or both haem groups contribute to the effect. However, mixing ATP with the cyanide complex of the oxidized enzyme leads to an optical perturbation somewhat different in amplitude and shape from that seen with the control. The similarities between the two difference spectra may suggest that cytochrome *a* is the primary chromophore perturbed by deprivation of Ca²⁺, in accordance with Saari *et al.* (1980). It is, however, of significance that with the cyanide complex the apparent dissociation constant for ATP

Table 2. Parameters used in the simulation of the observed rate constant, as in scheme (1) (see the Discussion section)

$k_{+1} (\text{s}^{-1})$	$k_{-1} (\text{s}^{-1})$	$10^{-4} \times K_2 (\text{M}^{-1})$
0.1	0.1	1.4
0.1	0.5	4
0.1	1.0	7.5
0.1	10.0	70.0

decreases from 0.1 to 0.085 mM. Following the hypothesis that the observed kinetics reflects dissociation of bound Ca^{2+} , this result implies that the binding site(s) on the cytoplasmic domains is controlled by the ligation state of the binuclear centre, showing interactions between the different functional domains in the enzyme.

ATP (and other anions) was found to modulate cytochrome *c* oxidase activity in pre-steady-state and steady-state kinetic experiments, both in solution and in COV. In fact, an increase and a subsequent decrease of activity were observed as a function of anion concentration (Kadenbach, 1986; Malatesta *et al.*, 1987). However, a correlation between optical perturbation and activity modulation induced by ATP is not straightforward, for the following reasons: (i) the concentration ranges are similar, but not identical; (ii) ATP modifies cytochrome *c* oxidase activity on a time scale faster than seconds, and therefore apparently faster than the event reported in this paper (limited to 0.1 s^{-1}).

However, the possibility cannot be ignored that, during turnover, an intermediate(s) with different binding parameters for Ca^{2+} may be populated.

Since ATP is in the millimolar concentration range in living cells, the experiments on the effect of Mg^{2+} on complex-formation are of physiological interest (also in view of the Mg -ATP complex prevailing *in vivo*). Mg^{2+} competitively subtracts ATP from binding to Ca^{2+} and thus leads to a decrease of the apparent affinity of complex-formation, although the kinetic parameters are unaffected and the observed effect could be simply due to competition between Ca^{2+} and Mg^{2+} for ATP.

Bisson *et al.* (1987) have shown that Mg^{2+} affects the control exerted by ATP on cytochrome *c* oxidase activity in steady-state conditions. Whether the two effects of Mg^{2+} (one on activity and the other on competition with Ca^{2+}) are indeed two aspects of the same phenomenon remains to be proven.

In conclusion, we can state that ATP is an allosteric effector of cytochrome *c* oxidase and that this effect occurs at ATP concentrations comparable with that prevailing under physiological conditions. The mechanism of control remains, however, to be explained, although it is possible that it is mediated by Ca^{2+} deprivation of cytochrome *c* oxidase.

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