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Effect of ATP on the activity of bovine heart mitochondrial b–c_1 complex

Michele Lorusso, Tiziana Cocco, Michele Minuto and Sergio Papa

Institute of Medical Biochemistry and Chemistry, University of Bari, Bari, Italy

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The effect of ATP on the reductase activity of purified bovine heart b–c_1 complex was studied. ATP stimulates the steady-state activity and the antimycin-insensitive pre-steady-state reduction of b and c_1 cytochromes, also causing changes of kinetic properties of the enzyme. There is no absolute specificity for ATP since other polyvalent anions such as EDTA and EGTA produce similar effects in the micromolar range. It is proposed that ATP stimulates the activity of the b–c_1 complex, chelating inhibitory cation(s), exerting a modulatory action on the enzyme.

Mitochondria; b–c_1 Complex; Electron transport; ATP; Complexing agent

1. INTRODUCTION

Electron flow in ubiquinol-cytochrome c reductase (b–c_1 complex or complex III; EC 1.10.2.2) of the mitochondrial respiratory chain is coupled to electrogenic proton translocation across the inner mitochondrial membrane.

The bovine heart b–c_1 complex shows invariably 11 polypeptide subunits [1], namely 8 supernumerary subunits in addition to those holding the two b hemes, heme c_1 and Rieske 2Fe-2S cluster. Attempts made to clarify the role of the supernumerary subunits suggest involvement of some of them in proton translocation and electron transfer activities [2–5]. Our group has recently shown that the complex exhibits a different susceptibility towards proteolytic digestion depending on the redox state of prosthetic groups [3]. These and related observations [6] indicate that the activity of the b–c_1 complex may be modulated by allosteric interactions of the constituent polypeptides.

It has been reported that anions, and in particular ATP, modulate the redox activity of mitochondrial cytochrome c oxidase, apparently by interacting with two supernumerary subunits (IV and VIII) [7–10].

In this paper we report on the effect of ATP on the activity of bovine b–c_1 complex. It is shown that ATP stimulates the reductase activity of the complex either in the soluble or in the membrane-reconstituted state by specifically accelerating the antimycin-insensitive reduction route of b and c_1 cytochromes.

Correspondence address: S. Papa, Institute of Medical Biochemistry and Chemistry, University of Bari, Piazza G. Cesare, 70124 Bari, Italy

Abbreviations: PMF, electrochemical proton gradient (proton motive force); DQH_2, durohydroquinone (duroquinol)

2. MATERIALS AND METHODS

2.1. *Enzyme and chemicals*

Ubiquinol-cytochrome c reductase was prepared from bovine heart mitochondria as in [11]. Horse heart cytochrome c (type VI), antimycin and soybean phospholipids (type II-b), were from Sigma Chemical Co. Duroquinol was from K&K Laboratories. Myxothiazol was from Boehringer Mannheim.

2.2. *Preparation of* b–c_1 *vesicles*

Reconstitution of b–c_1 complex into phospholipid vesicles (90% right-side oriented) was performed by cholate dialysis in 100 mM potassium-Hepes (pH 7.4) [12].

2.3. *Spectrophotometric determinations*

The reductase activity was measured with a dual-wavelength spectrophotometer at 550–540 nm in 100 mM K-Hepes/10 mM KCl (pH 7.2). The rates were corrected for the non-enzymatic reaction obtained either in the absence of the enzyme or with the enzyme inhibited by antimycin.

Redox changes of b and c_1 cytochromes were simultaneously followed at 10°C with an air turbine spectrophotometer at the wavelength couples of 562–540 nm and 550–540 nm, respectively.

3. RESULTS

Fig. 1 shows the influence of various anions on the reductase activity of cholate-solubilized b–c_1 complex. ATP and ADP induced a two-fold enhancement, at 10 and 20 mM, respectively, of the initial rate of cytochrome c reduction, with effectiveness appearing to be related to their charge. Inorganic phosphate also stimulated the reductase activity, its effect being maximal at 100 mM concentration (ionic strength, I = 0.23). Other inorganic anions such as chloride and sulphate exerted only a minor influence on the reductase activity.

Kinetic analysis (Table I) showed that ATP increased by 2–4-fold the apparent K_m and V_{max} of both duroquinol and cytochrome c without causing any deviation from linearity of the Lineweaver-Burk plots (not

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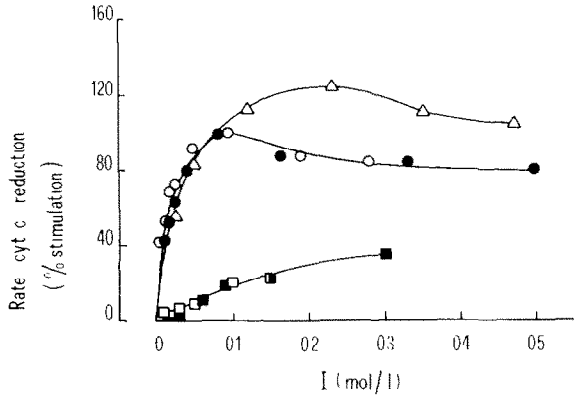

Fig. 1. Influence of increasing concentrations of anions on the activity of soluble b–c_1 complex. The reaction mixture contained 100 mM K-Hepes/10 mM KCl (pH 7.2), 0.02% Tween 80, 6 μM ferricytochrome c and 3 μg protein/ml of b–c_1 complex. The reaction was started by the addition of 33 μM duroquinol. The effectors were added as potassium salts in 100 mM Hepes (pH 7.2). (●) ATP; (○) ADP; (△) inorganic phosphate; (□) KCl; (■) K_2SO_4. Concentrations are reported on the abscissa after conversion into I (ionic strength).

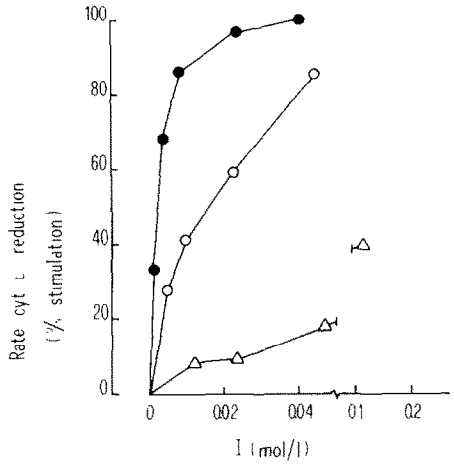

Fig. 2. Duroquinol-cytochrome c reductase activity in b–c_1 complex reconstituted into phospholipid vesicles. b–c_1 vesicles (22 μg protein/ml) were suspended in 100 mM K-Hepes/10 mM KCl (pH 7.2) also containing 6 μM ferricytochrome c and 0.15 μg/ml each valinomycin and nigericin; DQH_2, 33 μM. The specific activity in the control was 1.33 μmol·min^{-1}·mg protein^{-1}. (●) ATP, (○) ADP; (△) inorganic phosphate

shown). Similar results were obtained with saturating concentrations of ADP and inorganic phosphate.

The experiment of Fig. 2 shows the effect of externally added ATP, ADP and inorganic phosphate on the reductase activity of uncoupled b–c_1 vesicles. It appears that, upon insertion in the membrane, enzyme domain(s) exposed at the outer surface of the vesicles exhibits preferential sensitivity towards ATP with respect to ADP and inorganic phosphate. This can be of physiological significance by considering that ATP in the cell is in the millimolar concentration range and that the steady-state [ATP]/[ADP]·[P_i] ratio in the cytosol is much higher than in the matrix space [13].

The stimulatory effect of ATP on the reductase activity of the b–c_1 vesicles was influenced by the membrane potential, being maximal when the PMF was collapsed by the addition to the vesicle suspension of both valinomycin and nigericin (Fig. 3A).

The observed stimulatory effect of ATP is also produced by other non-physiological polyanions such as EDTA and EGTA. Fig. 3B shows, in fact, that EDTA at μM concentrations stimulated the reductase activity of the b–c_1 complex to the same extent as mM concentrations of ATP. Also the effect of EDTA was larger when the PMF was collapsed. Furthermore it was observed that additions of ATP in the presence of saturating concentrations of EDTA were ineffective. EDTA and EGTA caused the same changes in the kinetic properties of the enzyme (not shown) as those produced by ATP (Table I).

The reversibility of the ATP and EDTA effect was demonstrated as follows. b–c_1 vesicles either oxidized or reduced by duroquinol were incubated with saturating concentrations of ATP (3 mM) or EDTA (5 μM). The suspension was then passed through a Sephadex G-25 column. The reductase activity of the resulting b–c_1 vesicles, which returned to control values, was still two-fold stimulated by either ATP or EDTA.

Separate controls (see also Fig. 5) showed that Mg-ATP complex was as able as ATP in stimulating the reductase activity of the complex, neither was its effect influenced by the presence in the assay medium of 1 mM free Mg^{2+}.

Fig. 4 shows the effect of ATP on pre-steady-state reduction kinetics of b and c_1 cytochromes by duroquinol in b–c_1 vesicles supplemented with antimycin. In the presence of antimycin there was a two-fold stimulation of the apparent rate of reduction of b and c_1 cytochromes. Conversely, with myxothiazol present

Table I

DQH_2-cytochrome c reductase activity in soluble b–c_1 complex; influence of ATP on the apparent K_m and V_{max} values for duroquinol and cytochrome c

	(a) DQH_2		(b) Cyt. c	
	K_m	V_{max}	K_m	V_{max}
–	23	3.6	0.22	3.8
+ ATP	81	8.8	0.51	7.8

3 μg protein/ml b–c_1 complex were suspended in the reaction mixture also containing 0.02% Tween 80. Where indicated, 5 mM ATP was present. The K_m and V_{max} values were calculated by computer fitting from Lineweaver-Burk plots. In (a), DQH_2 concentration varied from 5.5 to 90 μM in the presence of 6 μM cytochrome c. In (b), the concentration of cytochrome c varied from 0.18 to 1.35 μM DQH_2 was used at 90 μM. Rates are expressed as μmol·min^{-1}·mg protein^{-1}. K_m are expressed as μM

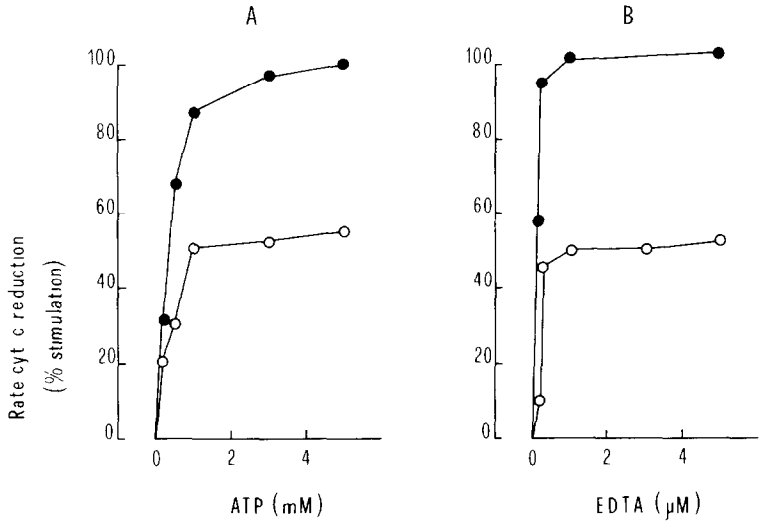

Fig. 3. Effect of ATP and EDTA on reductase activity of b–c_1 vesicles. The experimental conditions are those described in the legend to Fig. 2. For measurements of reductase activity in the coupled state, both valinomycin and nigericin were omitted. The specific activities in the control were 0.30 and 1.45 μmol·min^{-1}·mg protein^{-1} for the coupled and uncoupled state, respectively. Open symbols refer to the coupled state; closed symbols to the uncoupled state.

(not shown), ATP had no effect on the rate of b cytochrome reduction. The same results were obtained with 5 μM EDTA instead of ATP.

It has been shown that μM concentrations of Zn^{2+} ions inhibit the reductase activity of bovine mitochondrial b–c_1 complex ([14], see also [15,16]). The effect of Zn^{2+} ions on the activity of ATP stimulated b–c_1 vesicles is shown in Fig. 5A. Zn^{2+} at about 4 μM concentration completely reversed the stimulation of the reductase activity induced by 3 mM Mg-ATP in the presence of 1 mM $MgCl_2$. Higher concentrations of Zn^{2+} brought about a definite inhibition of the reductase activity. A complementary experiment (Fig. 5B) shows that Mg-ATP released the Zn^{2+}-dependent inhibition of the reductase activity leading, at higher con-

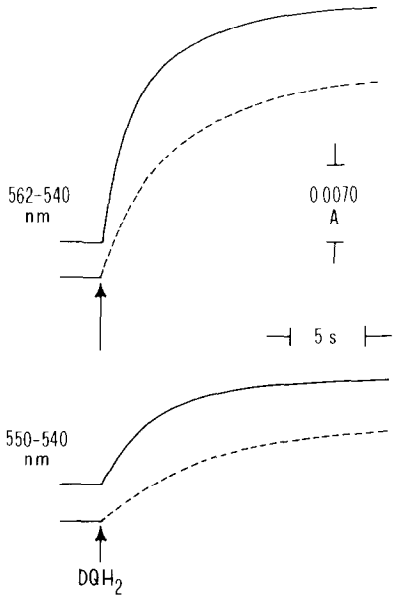

Fig. 4. Effect of ATP on pre-steady-state reduction kinetics of b and c_1 cytochromes in b–c_1 vesicles. The vesicles were suspended (final concentration 1 μM Cyt. c_1) in the reaction mixture described in the legend to Fig. 2 and supplemented with 5 μM antimycin. DQH_2 was used at 17 μM. Dotted line (control); solid line (+ 3 mM ATP).

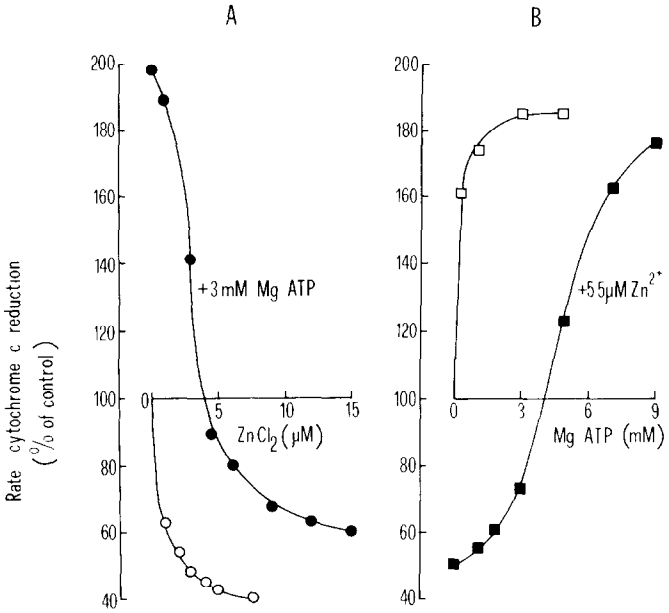

Fig. 5. Effect of Mg·ATP and $ZnCl_2$ on the reductase activity of b–c_1 vesicles. b–c_1 vesicles (22 μg protein/ml) were suspended in the reaction mixture described in the legend to Fig. 2 and supplemented with 1 mM $MgCl_2$. In Expt. A, $ZnCl_2$ was titrated in the absence (open symbols) and in the presence of 3 mM Mg-ATP. In Expt. B, Mg-ATP was added in the absence (open symbols) and in the presence of 5.5 μM $ZnCl_2$. Rates are expressed as percent of control values.

centrations, to a stimulation of the activity comparable to that observed in the absence of Zn^{2+}.

4. DISCUSSION

Interaction of mM concentrations of ATP with the bovine b–c_1 complex induces a marked stimulation of steady-state reductase activity, this being accompanied by changes of kinetic properties of the enzyme. Experiments with site-specific inhibitors (Fig. 4) indicate that ATP specifically affects the activity of the complex at the quinol oxidation site.

ATP is more effective in stimulating the activity of b–c_1 vesicles in the uncoupled than in coupled states. This would suggest that interaction of ATP with the b–c_1 complex affects a redox step different from that between b_{562} and cytochrome c_1 which has been shown to be controlled by PMF [17].

The stimulatory effect produced on the reconstituted enzyme by mM concentrations of ATP is also displayed by μM concentration of EDTA which is a typical metal chelator. These observations indicate that ATP enhances the reductase activity by chelating adventitious metal ions, which exert an inhibitory effect on the complex. It is, in fact, known that Zn^{2+} inhibits the second energy-coupling site of the respiratory chain [15,16] and that this effect can be reversed by ATP [16] (cf. Fig. 5).

The results presented would indicate the existence of mutually interacting binding site(s) for ATP (as well as non-physiological anions) and divalent heavy metals, in particular zinc. Mutually exclusive binding of adenine nucleotides and Zn^{2+} ions appears to exert a modulatory effect on the redox activity of the enzyme at the quinol oxidation site.

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