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INACTIVE TO ACTIVE TRANSITIONS OF THE MITOCHONDRIAL ATPase COMPLEX AS CONTROLLED BY THE ATPase INHIBITOR

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Summary

The hydrolytic and phosphorylation activities of the ATPase complex of bovine heart mitochondria are regulated by the ATPase inhibitor of Pullman and Monroy [1]. The inhibiting action of the peptide on ATPase activity can be overcome by a proton-motive force. Submitochondrial particles that contain the inhibitor, either intrinsically or externally added, show a lag that precedes phosphorylation. Particles devoid of the inhibitor, or particles that are in an 'active' state fail to present the lag. Accordingly, the data indicate that, prior to the onset of phosphorylation, the ATPase complex undergoes a transition to an active state through a process that involves the inhibitor. The transition depends on the concentration of ATP, 50 μM ATP giving 50% inhibition of the proton-motive force-induced transition.

Introduction

A peptide that inhibits the ATPase activity of soluble and particulate mitochondrial F_1-ATPase was originally isolated by Pullman and Monroy [1]. A regulatory function of the inhibitor has been proposed [2—5], but its precise role in mitochondrial oxidative phosphorylation is still unknown. In this work, the action of the inhibitor on the ATPase and phosphorylation reactions of

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Abbreviations: F_1-ATPase, soluble mitochondrial ATPase; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; MES, 2-(*N*-morpholino)ethanesulfonic acid.

submitochondrial particles has been further examined. The results indicate that the inhibitor senses the potential of the membrane and the adenine nucleotide composition of the environment in contact with F_1-ATPase. Depending on these two factors, the inhibitor is apparently involved in the regulation of a reversible inactive to active transition of the ATPase complex.

Materials and Methods

MgATP particles [6], EDTA particles [6], State 3 particles [4], and Sephadex particles [7] from beef heart mitochondria were prepared as indicated in the respective references. The preparation of the inhibitor has also been described [8]. The binding of the inhibitor to inhibitor-depleted particles was carried out by incubating 5 mg of submitochondrial particles with 30 μg of inhibitor protein in 0.5 ml of 10 mM Tris/Mes buffer, pH 6.5, 250 mM sucrose, and 0.5 mM MgATP for 10 min at 30°C. At this time 0.5 ml of 0.25 M sucrose was added and the particles were centrifuged at 45 000 rev./min (Spinco 50 Ti rotor) for 15 min. The pellet was suspended in 0.25 M sucrose/5 mM $MgSO_4$ and used for further studies. Phosphorylation rates were determined in the presence of an ATP-trapping system, 170 mM sucrose, 50 mM Tris/acetate, pH 7.5, 3 mM magnesium acetate, 8 mM potassium phosphate (pH 7.5), 20 mM glucose, 5 mM succinate, 0.5 mM NADP, and seven units of hexokinase and glucose-6-phosphate dehydrogenase. The reduction of NADP at 340 nm was taken as an index of ATP formation. Under our experimental conditions no NADPH oxidation was detected. ATPase activity was measured in the presence of an ATP-regenerating system [9]. More experimental details are given in the respective tables and figures.

Results

The ATPase activity of soluble and particulate F_1-ATPase is easily inhibited by the inhibitor, provided the enzyme is incubated with the inhibitor at a relatively acid pH and in the presence of Mg^{2+} and ATP [1]. In the soluble enzyme, this inhibition is not easily reversed by dilution, exchange of adenine nucleotides, and removal of bound ATP (Nordenbrand, K. and Ernster, L., unpublished results). On the other hand, in submitochondrial particles Van de Stadt et al. [4] reported activation of ATP hydrolysis (presumably through removal of inhibitor from F_1-ATPase) upon establishment of electron transport.

Table I shows that particles that contain inhibitor (MgATP particles) are activated by succinate, while those that are depleted of inhibitor (State 3 and EDTA-Sephadex particles) fail to respond to succinate. Moreover, these particles are characterized by a relatively high ATPase activity. That inhibitor is indeed involved in the succinate effect on ATPase activity is confirmed by the observation that in particles to which the inhibitor is added back (EDTA-Sephadex-inhibitor particles) the response to succinate is restored.

Some of the characteristics of the inhibitor-dependent inactive to active transition of the ATPase of MgATP particles are illustrated in Table II. FCCP and malonate added after electron transport has been established do not change

TABLE I

EFFECT OF SUCCINATE ON THE ATPase ACTIVITY OF SUBMITOCHONDRIAL PARTICLES WITH DIFFERENT CONTENTS OF ATPase INHIBITOR

The indicated particles (100 μg) were incubated in the standard mixture for measurement of ATPase activity [7] that also contained 5 mM succinate where shown. The mixture was incubated for 4 min, at which time 3 mM ATP was added to start the reaction, and subsequently 3 μM FCCP was added.

Particles	Succinate addition 4 min before ATP	ATPase activity (nmol · min^{-1} · mg^{-1})	
		—FCCP	+FCCP (after ATP)
MgATP	—	120	260
MgATP	+	207	665
State 3	—	443	1374
State 3	+	458	1422
EDTA-Sephadex	—	3860	3860
EDTA-Sephadex	+	3780	3780
EDTA-Sephadex	—	102	102
EDTA-Sephadex	+	395	482

the activating action of succinate on ATP hydrolysis. However, FCCP or malonate added before succinate effectively prevent the effect of succinate. These results imply that the activating action of succinate may be prevented, but not reversed, by a collapse of the membrane potential or by inhibition of electron transport.

TABLE II

EFFECT OF THE ORDER OF ADDITION OF MALONATE AND FCCP (EXPT. A) AND NIGERICIN AND VALINOMYCIN (EXPT. B) ON THE ACTION OF SUCCINATE ON THE ATPase ACTIVITY OF MgATP PARTICLES

MgATP particles (100 μg) were incubated with 5 mM succinate, and after 4 min 1.6 mM malonate or 3 μM FCCP was added. After 1 min ATP was introduced and the ATPase activity recorded, FCCP was added as shown. In the second experiment, particles were incubated with 1.5 mM malonate or 3 μM FCCP for 1 min. This was followed by 5 mM succinate for 4 min, at which time ATP was added to start the reaction. In Expt. B, particles were added to a mixture that contained 5 mM succinate and 0.01 μM nigericin or valinomycin and incubated for 4 min before ATP was introduced. The rest of the experimental conditions as described in Table I.

	ATP hydrolyzed (nmol · m^{-1} · mg^{-1})	
	—FCCP	+FCCP
Expt. A		
Addition after succinate		
—	216	559
Malonate	226	579
FCCP	—	554
Addition before succinate		
—	216	559
Malonate	154	202
FCCP	—	241
Expt. B		
Additions		
—	207	665
Nigericin	154	465
Valinomycin	178	352
Nigericin + valinomycin	183	183

Table II (Expt. B) shows that nigericin (+ K^+), which equilibrates ΔpH across membranes, induces a partial inhibition of the action of succinate on ATPase activity, and that nigericin in combination with valinomycin (a neutral K^+ ionophore) completely prevents the action of succinate. Thus it appears that both ΔpH and $\Delta\psi$ are required in the activation of ATP hydrolysis by succinate.

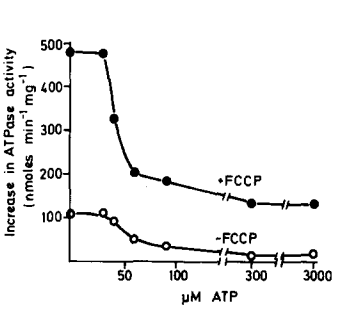
It is interesting to point out that the activation of the ATPase complex by succinate is prevented by ATP (Fig. 1). Approximately 50 μM ATP induces half-maximal inhibition of the activating action of succinate. This set of results would seem to indicate that the transition from the inactive to the active state of the ATPase complex is regulated by the membrane potntial and the adenine nucleotide composition of the medium.

Effect of the inhibitor on oxidative phosphorylation

The afore-mentioned data indicate that the establishment of the protonmotive force in the membrane induces higher ATPase rates through a process that is regulated by the inhibitor. The possibility of a similar control of the synthetic activity of the ATPase was also explored.

Fig. 2 shows the rates of phosphorylation and ATPase activities of MgATP particles preincubated for various intervals of time with succinate before phosphorylation or hydrolysis is started (by the addition of ADP or ATP). As the time of incubation is prolonged, the rate of phosphorylation decreases, whilst that of ATPase activity increases. It may also be observed that at an incubation time of 1 min, the rate of phosphorylation is similar to the ATPase rate with FCCP. At longer times, the ATPase rate becomes significantly higher.

The pattern of phosphorylation of MgATP particles added to a mixture that contains ADP and succinate is shown in Fig. 3. In our conditions phosphoryla-



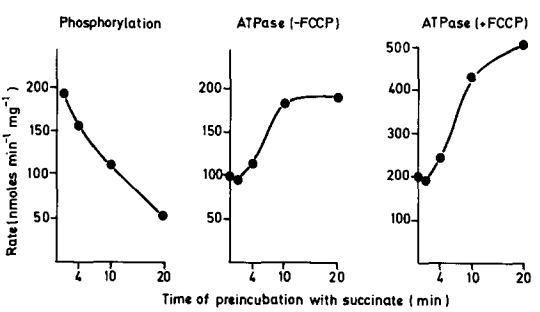


Fig. 1. Effect of ATP on the stimulating action of succinate on the ATPase activity of MgATP particles. The particles (100 μg) were incubated with the indicated concentrations of ATP for 1 min, at this time 5 mM succinate was added and the incubation continued for another 4 min. 3 mM ATP was added and the ATPase activity recorded, subsequently 3 μM FCCP was introduced. In the experiment with 3 mM ATP, the second addition of ATP was not made. The standard incubation mixture that contains an ATP-regenerating system was employed. The stimulating action of succinate on the ATPase activity is plotted. In the absence of succinate the activity was 108 nmol · min^{-1} · mg^{-1}.

Fig. 2. Phosphorylation and ATPase rates of MgATP particles incubated with succinate for various times. Phosphorylation and ATPase rates (±3 μM FCCP) were measured as described in Material and Methods, except that the particles (100 μg) were incubated prior to the addition of 0.33 mM ADP or 3 mM ATP with 5 mM succinate for the indicated times.

tion does not become apparent until about 15 s have elapsed. This would indicate that prior to the onset of phosphorylation, the ATPase complex exists in an inactive state. Upon establishment of a membrane potential (as supported by electron transport), the ATPase undergoes a transition to the active phosphorylating state.

The lag that precedes phosphorylation is not observed in particles previously exposed to succinate (Fig. 3B), nor in particles devoid of the inhibitor (Fig. 3C). Further evidence for the control of phosphorylation by the inhibitor is shown in Fig. 4. The phosphorylation pattern of particles devoid of inhibitor and of those in which the inhibitor was added back requires and extremely long period before steady-state phosphorylation rates are established. This does not occur in Sephadex particles. It should be emphasized that there is an important difference in the time required for the activation of the ATPase complex in the MgATP particles and in the reconstituted particles (compare Fig. 1A to Fig. 4A). This indicates that some factors involved in the interaction of the inhibitor with F_1-ATPase may control the rate at which the ATPase complex is activated. This problem is currently investigated.

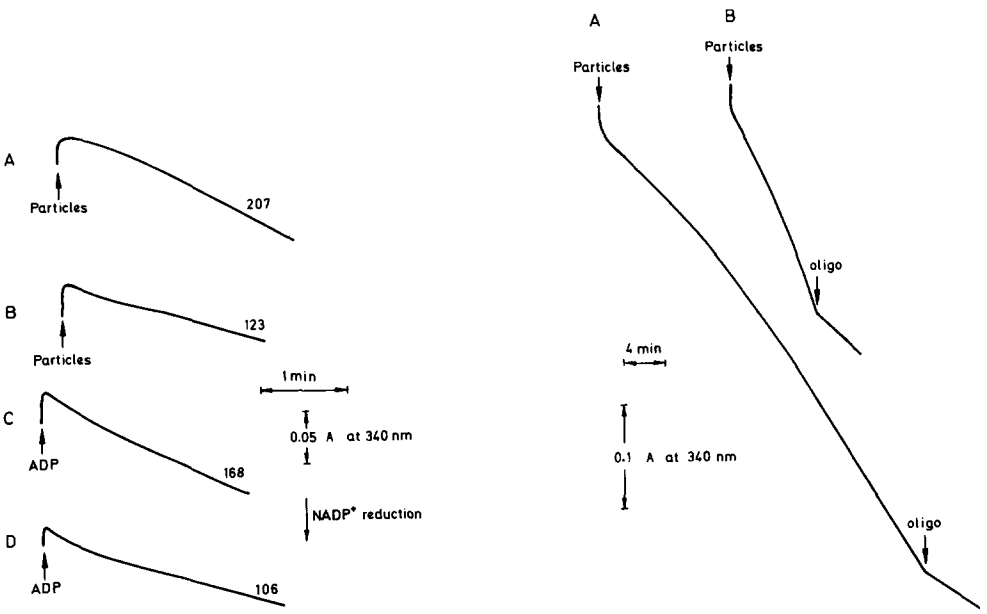


Fig. 3. Phosphorylation pattern of MgATP and State 3 particles. The experimental conditions were as in Fig. 2 (100 μg particle protein). In (A) MgATP particles were added to mixtures that contained 0.33 mM ADP and 5 mM succinate. (B) The conditions were as in (A), except that State 3 particles were added. In (C) MgATP particles were incubated for 4 min with succinate, at this time 0.33 mM ADP was added and ATP formation recorded. (D) The conditions were as in (C), except that State 3 particles were used. The numbers on the side of the trace show the steady-state phosphorylation rates (NADP reduced, nmol · min^{-1} · mg^{-1}). Myokinase activity (60 nmol · min^{-1} · mg^{-1}) was substracted from the experimentally observed values of ATP formation.

Fig. 4. Phosphorylation pattern of EDTA-Sephadex particles and EDTA-Sephadex particles treated with inhibitor. The experimental conditions were as in Fig. 3, except that EDTA-Sephadex particles treated with inhibitor and EDTA-Sephadex particles (100 μg protein each) were added in (A) and (B), respectively, to mixtures that contain ADP, succinate, and 0.05 μg oligomycin/1 mg of particle protein. At the second arrow 5 μg oligomycin was added to the incubation mixture.

Conclusions

Two main conclusions can be drawn from the experimental data presented:

(1) The ATPase complex may exist in an inactive state, both in the hydrolytic and phosphorylating direction.

(2) Upon the establishment of a membrane potential, and prior to the formation of ATP, the complex undergoes a transition to the active state through a process that is signaled by the inhibitor.

These two conclusions would be in agreement with the studies of Harris and Crofts [10] on photosynthetic phosphorylation. These authors also observed a lag that preceded phosphorylation and which correlated with ATPase rates.

Most likely the inhibitor is not directly involved in the opening of the H^+ channel. MgATP particles with a respiratory control of 3.1 (to FCCP), and an ATPase activity of 0.16 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1}, upon passage through a Sephadex column showed an ATPase activity of 1.4 and a respiratory control of 2.4. Apparently the removal of the inhibitor does not increase the permeability of the membrane to H^+. Several attempts were made to explore whether inhibitor is required in the phosphorylation reaction: the results were negative. For instance, the steady-state phosphorylation rates of EDTA-Sephadex, and inhibitor-treated Sephadex particles, with ATPase activities of 670, 2800, and 72, were 69, 75 and 75 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1}, respectively.

Nevertheless the data show that the inhibitor inhibits the hydrolytic and synthetic activities of the ATPase complex by acting on its F_1 component. Therefore it is likely that the peptide controls the enzymic activity of the ATPase complex by a reversible modification of the catalytic site. This modification depends on the membrane potential as a driving force for the activation of the inhibitor-F_1-ATPase complex, and on the concentration of ATP which would seem to exert two actions, i.e. to prevent the transition to the active state (Fig. 1) and to induce inactivation of the enzyme.

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