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THE INTERACTION BETWEEN THE MITOCHONDRIAL ATPase (F_1) AND THE ATPase INHIBITOR

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SUMMARY

1. The naturally occurring mitochondrial ATPase inhibitor inhibits the mitochondrial ATPase (F_1) non-competitively.

2. The interaction between inhibitor and inhibitor-depleted F_1 or submitochondrial particles is diminished when the ratio of ATP/ADP is low or when energy is generated by substrate oxidation.

3. The dissociation of the inhibitor from coupled Mg–ATP particles is promoted when substrates are being oxidized. This results in the appearance of a large uncoupler-stimulated ATPase activity. Activation of the uncoupler-stimulated ATPase activity is also achieved by incubation of the particles with ADP.

4. The ATPase activity of Mg–ATP particles is determined by the turnover capacity of F_1. When endogenous inhibitor is removed, energy dissipation becomes the rate-limiting step. This energy dissipation can be activated by an uncoupler.

5. Evidence is presented for the existence of a non-inhibited intermediate F_1–inhibitor complex.

INTRODUCTION

The mitochondrial ATPase (F_1) has been isolated from beef-heart[1] and purified to homogeneity (see refs 1–5). The molecular weight is 360000 (ref. 2). Pullman and Monroy[6] described the isolation and properties of a naturally occurring ATPase inhibitor derived from beef-heart mitochondria. The inhibitor, a trypsin-sensitive protein, of mol. wt 10500 (refs 4 and 7), strongly suppresses the ATPase activity of both isolated F_1 (ref. 6) and inhibitor-depleted AS particles[4].

Horstman and Racker[4] have shown that for binding of the inhibitor to F_1 or inhibitor-depleted AS particles Mg^{2+} and ATP are necessary.

Abbreviations: F_1, mitochondrial ATPase; A particles, submitochondrial particles prepared by sonication of beef-heart mitochondria in an ammonia solution at pH 9.2; AS particles, submitochondrial particles prepared by treatment of A particles with Sephadex G-50; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; 1799, bis-hexafluoroacetone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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Ernster and coworkers[8,9], using "EDTA particles", obtained evidence that the ATPase inhibitor is a unidirectional inhibitor of F_1, *i.e.* it inhibits all ATP-driven processes in submitochondrial particles, without affecting oxidative phosphorylation. Furthermore, the inhibitor leaves the extent of the oligomycin-induced "respiratory control" unaffected. The inhibitor could not replace oligomycin in promoting oxidative phosphorylation in uncoupled "EDTA particles". The authors suggested that the ATPase inhibitor has an important role as a directional regulator of respiratory chain-linked energy transfer, controlling the back flow of energy from ATP to the mitochondrial electron- and ion-transport systems. Evidence in support of this view is presented in this paper.

METHODS AND MATERIALS

Preparation of beef-heart mitochondria, submitochondrial particles and coupling factors

Beef-heart mitochondria[10], A particles[11], coupling factor F_1 (ref. 12), the ATPase inhibitor[4] and Mg–ATP particles[13], were prepared according to the published procedures. AS particles[4] are virtually devoid of endogenous ATPase inhibitor. The F_1 preparation used also contains negligible amounts of inhibitor[7].

Measurement of ATPase activity and oxygen uptake

ATPase activities, including the measurement of initial rates, were determined by the change of pH of the reaction medium with a sensitive pH measuring system (Electronic Instrument Ltd.), including a combined micro-glass electrode (Gm 23), a pH-measuring unit (C33B-2) and a Vibron electrometer (33B-2).

Oxygen uptake was monitored in the same vessel with a Teflon-covered Clark oxygen electrode (Yellow Springs Instr.) with appropriate polarization circuitry.

The reaction mixture contained at 30 °C, in a final volume of 1.5 ml, 0.375 mmole sucrose, 2.5 μmoles ATP and 1.25 μmoles $MgCl_2$ (added from a stock solution containing 100 mM ATP and 50 mM $MgCl_2$ at pH 6.8), 4 μmoles potassium phosphate buffer (pH 6.9) and 0.75 mg defatted bovine serum albumin, unless stated otherwise in the legends. ATPase measurements were started by the addition of the particles or of F_1. In all other cases Mg^{2+}–ATP, Mg^{2+}–ADP and Mg^{2+}–AMP were added from equimolar stock solutions containing the adenine nucleotides and $MgCl_2$ at pH 6.8. The electrode responses were calibrated with standard solutions of NaOH or oxalic acid.

Reconstitution of submitochondrial particles with ATPase inhibitor

A sample of submitochondrial particles (usually 10 mg of protein) was incubated with an appropriate amount of ATPase inhibitor in a final volume of 5 ml containing 1.25 mmoles sucrose, 50 μmoles Tris–*N*-tris(hydroxymethyl)-methyl-2-aminoethanesulphonic acid) (TES) buffer (pH 6.7) and 2.5 μmoles Mg^{2+}–ATP. Extra additions or omissions are indicated in the legends. After 20 min at 25 °C the submitochondrial particles were collected by centrifugation at $130000 \times g$ for 15 min at 4 °C. The particles were washed once and resuspended in 0.25 M sucrose.

Reconstitution of F_1 with ATPase inhibitor

A sample of F_1 (usually 0.5 mg of protein) was incubated with an appropriate amount of inhibitor in a final volume of 2 ml containing 0.5 mmole sucrose, 20 μmoles Tris–TES buffer (pH 6.7) and 1 μmole Mg^{2+}–ATP. After 20 min at 25 °C the F_1 was collected by precipitation with an equal volume of saturated $(NH_4)_2SO_4$ (pH 7.2). The precipitate obtained after centrifugation in the cold (10 min at 20000 × *g*) was stored as a 0.5% solution in 0.25 M sucrose, 0.01 M Tris–sulphate buffer (pH 7.4) and 0.001 M EDTA and kept at room temperature.

Centrifugation of Mg–ATP particles under dissociating conditions

A sample of Mg–ATP particles (usually 3–4 mg of protein) was incubated at 25 °C for 1 min in 6 ml of a mixture containing the components described in the legends to the figures. Then the entire suspension was centrifuged at 200000 × *g* for 10 min as quickly as possible (the rotor of the MSE-50 superspeed centrifuge reached 200000 × *g* within 3 min). The collected particles were washed once and resuspended in 0.25 M sucrose.

Construction of the time course of inhibition of the ATPase by the ATPase inhibitor

To a mixture of 1 ml containing 0.25 mmole sucrose, 5 μmoles Tris–TES buffer (pH 6.9) and 1 μmole Mg^{2+}–ATP, the amounts of Mg^{2+}–ADP, Mg^{2+}–AMP or other components indicated in the legends were added. Then the ATPase reaction was started by the addition of AS particles or F_1 at 25 °C. The rate of proton production declines slowly because of the formation of ADP. ADP is a competitive inhibitor of the ATPase reaction[14]. Immediately after this experiment, in an identical run, a 5-fold excess of ATPase inhibitor was introduced into the reaction vessel 5 s after starting the ATPase reaction. The proton production rapidly decreases when the inhibitor is bound to the ATPase. Because the inhibition by the inhibitor is non-competitive (see Table I) one can correct for the ADP effect by correlating the rates of proton production at the horizontal intersection points of the two curves (at these points the amount of ADP formed is the same in both curves). The percentage inhibition of the ATPase activity caused by the binding of the inhibitor only can now be calculated.

Analytical methods

Soluble protein was determined by the method of Lowry *et al.*[15] with bovine serum albumin as a standard, insoluble protein by the biuret method as described by Cleland and Slater[16].

Materials

NAD^+, alcohol dehydrogenase, hexokinase, ATP, ADP and AMP were obtained from Boehringer und Söhne. Oligomycin was kindly provided by the Upjohn Chemical Co., the uncouplers bis-hexafluoroacetone (1799) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were a gift of Dr P. Heytler. TES was purchased from Calbiochem. Crystalline bovine serum albumin was defatted according to Goodman[17]. All other chemicals were of analytical-grade purity. The enzymes were dialysed before use against an excess of 10 mM Tris–

acetate buffer (pH 7.4) for 3 h. All acids used in the reaction mixtures were neutralized with Tris.

RESULTS

Non-competitive inhibition of the ATPase reaction by the inhibitor

With partially-inhibiting amounts of the inhibitor bound to either AS particles or F_1, the apparent kinetic parameters of the ATPase reaction were determined (Table I). Neither the K_m for ATP nor the K_i for ADP are changed upon binding of the inhibitor to AS particles or F_1. The effect of the inhibitor is clearly of the non-competitive type. Similar results were reported by Juntti *et al.*[9] with "EDTA particles". It is to be noted that the affinity of F_1 for ATP is lower than that of the membrane-bound ATPase, while the affinity of F_1 for ADP is higher than that of submitochondrial particles (see also ref. 14). As a result the ATPase activity of F_1 is more strongly inhibited by the ADP formed during the reaction than that of submitochondrial particles.

TABLE I

NON-COMPETITIVE INHIBITION OF THE ATPase REACTION BY THE ATPase INHIBITOR

5 mg of AS particles were incubated with 2 or 3 μg inhibitor, as described under Methods. 0.5 mg F_1 was incubated with 6 μg inhibitor as described under Methods. Initial ATPase activities were recorded at 25 °C by starting the reaction with 50 μg of AS particles or 4 μg F_1. The reaction mixture contained in a final volume of 1 ml 250 μmoles sucrose, 5 μmoles Tris–acetate buffer (pH 7.4), the concentration of Mg^{2+}–ADP as indicated in the table and concentrations of Mg^{2+}–ATP, ranging from 0.7 to 10 mM. The initial velocities were plotted in a double reciprocal plot against ATP concentration and analysed by computer. The apparent kinetic parameters are given in the Table. Correlation coefficients are better than 0.99.

Prepn	*Mg^{2+}–ADP (mM)*	*Inhibitor (μg/mg)*	*K_mATP app. (mM)*	*K_iADP app. (mM)*	*V (μequiv H^+/min per mg)*
AS	0	0	0.70	—	3.7
particles	0.5	0	—	0.17	3.5
	0	0.4	0.71	—	1.8
	0.5	0.4	—	0.15	1.9
	0	0.6	0.72	—	1.2
	0.5	0.6	—	0.15	1.3
F_1	0	0	1.25	—	263
	0.25	0	—	0.10	255
	0	12	1.23	—	52
	0.25	12	—	0.11	54

The binding of the inhibitor to F_1 or AS particles

The binding of the inhibitor to F_1 or AS particles can be followed by comparison of the continuous decrease in ATPase activity with a parallel experiment without the addition of the inhibitor, as discussed in Methods. In Fig. 1 it is shown that the rate of binding of the inhibitor to AS particles or to F_1 is greatly diminished

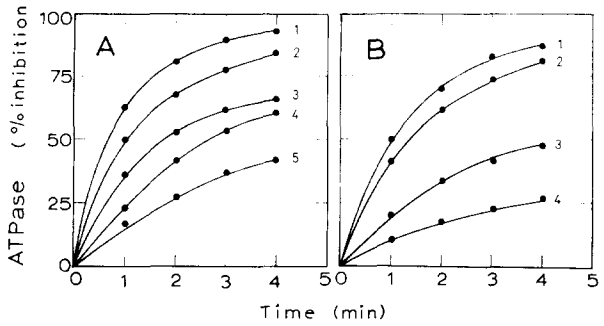


Fig. 1. Time course of inhibition of the ATPase by an excess of ATPase inhibitor. Incubation conditions and calculation as described under Methods. (A) The reaction was started with 0.15 mg of AS particles and 0.8 μg of inhibitor. Further additions: Curve 1, none; Curve 2, 2 mM Mg^{2+}–AMP; Curve 3, 0.5 mM Mg^{2+}–ADP; Curve 4, 1 mM Mg^{2+}–ADP; Curve 5, 2 mM Mg^{2+}–ADP. (B) The reaction was started with 2 μg of F_1 and 0.5 μg of inhibitor. Further additions: Curve 1, none; Curve 2, 1 mM Mg^{2+}–AMP; Curve 3, 0.5 mM Mg^{2+}–ADP; Curve 4, 1 mM Mg^{2+}–ADP.

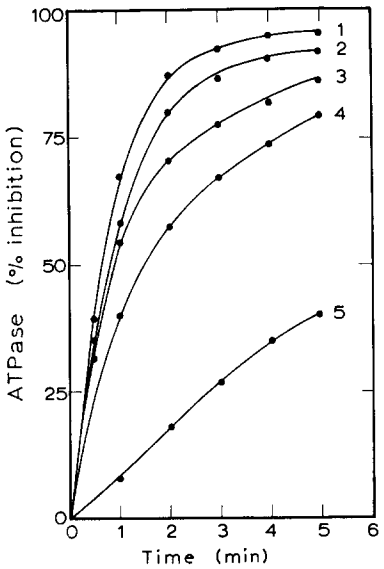
when the ratio of ADP/ATP is high. Also the final extent of the inhibition is considerably smaller (not shown) than in the control experiment. In one experiment Mg^{2+}–AMP was added instead of Mg^{2+}–ADP to determine the effect of salt on the F_1-inhibitor interaction, since Horstman and Racker[4] reported that the F_1–inhibitor interaction is strongly diminished by 50 mM KCl. It is obvious that this addition has much less effect.

After having found that the rate of binding of inhibitor to the ATPase is influenced by the ATP/ADP ratio we decided to investigate whether the inhibition of the ATPase reaction by the inhibitor is also influenced when energy is supplied to the particles by respiratory activity. It can be seen in Fig. 2 that in particles (recoupled with a low concentration of oligomycin) oxidizing NADH the reaction between F_1 and inhibitor is strongly inhibited. This effect is abolished by antimycin or by omitting oligomycin. As shown by Curve 5 in Fig. 2 the much slower (coupled) oxidation of succinate does not have the same effect as NADH oxidation.

From the data presented above it is concluded that the interaction between inhibitor-free F_1 and inhibitor is strongly weakened when either the ratio of ADP/ATP is high or when oxidative energy is supplied. The effect of phosphate (up to 5 mM) on the F_1–inhibitor interaction was rather small and not different from control experiments, where sulphate was added instead of phosphate.

Studies on the binding of inhibitor to A particles

Uncoupled A particles still have some endogenous inhibitor bound to F_1, although the ATPase activity is very high (approximately 10 times higher than that of Mg–ATP particles). This is shown by Curve 1 of Fig. 3. When the A particles were incubated with an excess of inhibitor in the absence of Mg^{2+}–ATP, only a part of the added inhibitor was bound. The ATPase activity was inhibited by 65% (Curve 2). When these particles were re-incubated with Mg^{2+}–ATP, the inhibition of the ATPase activity was markedly increased, suggesting that a considerable part of the inhibitor was bound to the particles in a non-inhibitory state (Curve 6).



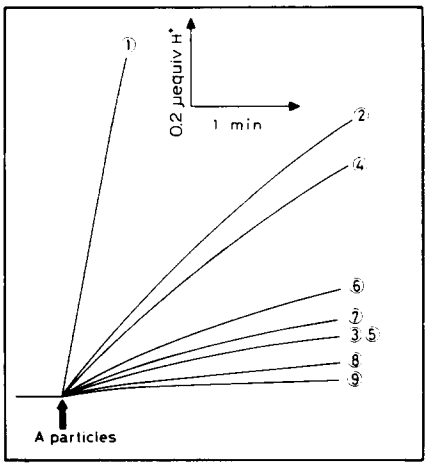


Fig. 2. Effect of substrate oxidation on the interaction between inhibitor and AS particles. Incubation conditions and calculation as described under Methods. The reaction was started with 0.3 mg of AS particles and 2 μg of inhibitor. The reaction mixture contained in a final volume of 1 ml at 25 °C, 0.25 mmole sucrose, 2.5 μmoles Tris–TES buffer (pH 6.9), 2 μmoles Mg^{2+}–ATP, 2.5 μmoles semicarbazide, 100 μmoles ethanol and 100 μg of dialysed alcohol dehydrogenase. Further additions: Curve 1, none; Curve 2, 0.5 μmole NADH; Curve 3, 0.08 μg of oligomycin and 5 μmoles succinate, instead of the complete NADH regenerating system; Curve 4, 0.5 μmole NADH, 0.08 μg of oligomycin and 0.15 μg of antimycin; Curve 5, 0.5 μmole NADH and 0.08 μg of oligomycin.

Fig. 3. The interaction between inhibitor and A particles. 8 mg of A particles are preincubated with 8 μg of inhibitor and isolated as described in Methods. The ATPase activity of 0.25 mg of the isolated particles was tested as described in Methods. Further additions or omissions in the preincubation mixture: Curve 1, without inhibitor; Curve 2, without Mg^{2+}–ATP; Curve 3, none; Curve 4, without Mg^{2+}–ATP, *plus* 25 μM 1799; Curve 5, *plus* 25 μM 1799; Curve 6, particles, obtained from Curve 2 were re-incubated with Mg^{2+}–ATP before assay; Curve 7 particles, obtained from Curve 2 were re-incubated with Mg^{2+}–ATP *plus* 30 μM 1799; Curve 8 particles, obtained from Curve 3, were re-incubated with Mg^{2+}–ATP *plus* 30 μM 1799; Curve 9, particles, obtained from Curve 5, were re-incubated with Mg^{2+}–ATP *plus* 30 μM 1799.

When the particles were incubated with inhibitor in the presence of Mg^{2+}–ATP, the ATPase activity was inhibited by about 90% (Curve 3). When these particles were re-incubated, however, in the presence of Mg^{2+}–ATP *plus* an uncoupler, the ATPase activity was inhibited even more (Curve 8), suggesting again that a minor part of the inhibitor in this case was bound to the particles in a non-inhibitory state. That an uncoupler is necessary to obtain complete inhibition by the inhibitor bound to F_1, follows also from later experiments (see also Curves 7 and 9).

From these experiments, it can be concluded that conditions exist where the inhibitor is already bound to the particles but in a non-inhibitory form. Binding of the inhibitor to F_1 to form the inhibited state requires Mg^{2+}–ATP, as was already shown by Horstman and Racker[4]; this process is insensitive to an uncoupler.

Transition of the uninhibited state to the inhibited state is promoted most effectively by Mg^{2+}–ATP *plus* an uncoupler.

Studies on the dissociation of the inhibitor from Mg–ATP particles

To study the dissociation of the inhibitor bound to F_1, we used tightly coupled Mg–ATP particles, at a relatively low pH (6.9) in the presence of defatted bovine serum albumin and phosphate. In our experiments the pH never changed by more than 0.1 unit, a change that will not considerably influence the interaction of inhibitor and F_1 (*cf.* ref. 4). These particles contain a considerable amount of endogenous inhibitor. Under the conditions employed here, the particles exhibited an ADP-induced respiratory-control index of 1.65 with succinate as the substrate.

In Fig. 4 the ATPase activity of the Mg–ATP particles is shown under standard conditions (Curve A). The ATPase activity is considerably enhanced by the phosphate present in the reaction mixture (see also ref. 18). When the uncoupler 1799 is added together with the particles, hardly any stimulation of the ATPase activity is observed. Apparently, the rate-limiting step in the overall reaction is the turnover capacity of F_1. When during the ATPase reaction more and more ADP is formed, the uncoupler progressively stimulates the ATPase activity (Curves A2–4). This effect is also obtained by preincubation of the particles in the presence

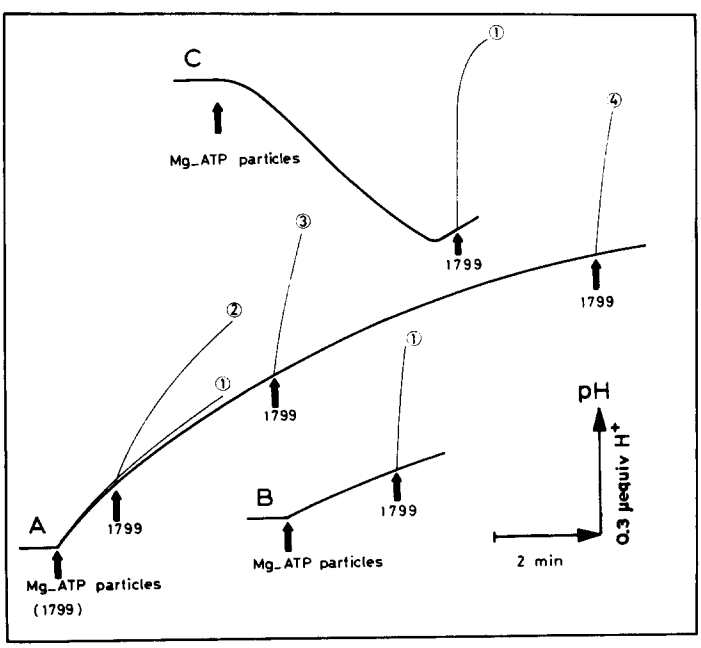


Fig. 4. Activation of the uncoupler-stimulated ATPase activity in Mg–ATP particles by ADP and succinate oxidation. ATPase activity was measured at 30 °C as described in Methods. A, Curves 1–4, 15 μM 1799 was added after the ATPase reaction had lasted 0, 1, 4 and 10 min, respectively. B. 5 mM succinate and 1 mM Mg^{2+}–ADP were added together with the particles; Curve 1, 15 μM 1799 was added 2 min after the ATPase reaction was started. C, the same as under B, but with the omission of Mg^{2+}–ATP. Under these conditions oxidative phosphorylation occurs and the formation of ATP is reflected in a continuous and substantial alkalinization of the medium; Curve 1, 20 s after anaerobiosis 15 μM 1799 was added.

of Mg^{2+}–ATP *plus* Mg^{2+}–ADP. Preincubation is necessary, for when the uncoupler is added together with the particles absolutely no stimulation of the ATPase activity is seen. When the particles are preincubated with succinate, Mg^{2+}–ADP and Mg^{2+}–ATP, the uncoupler-stimulated ATPase activity is even more enhanced (Curve B1).

It should be noted that the activation by ADP of the uncoupler-stimulated ATPase activity is apparently inhibited by uncoupler, because when the uncoupler is added shortly after the particles (Curves A1,2) the ATPase activity does not increase any more when progressively more ADP is formed; on the contrary, the expected competitive inhibition by the ADP generated is shown. Thus, the ADP effect is only seen under coupled conditions.

Incubation of the particles under State-3 conditions, in the absence of added Mg^{2+}–ATP, is accompanied by a high phosphorylation rate (Curve C, 107 nequiv H^+/min per mg protein). Oxygen uptake was 130 natoms O/min per mg protein. Using a value of 0.6 equiv H^+/equiv of ATP formed (Bertina, R.M., personal communication), a P/O ratio of 1.4 can be calculated, with succinate as a substrate. For experimental reasons, only succinate oxidation was used in this experiment and not NADH, because no pH changes in the medium should be observed at all during the oxidation when Mg^{2+}–ATP is omitted. After the particles had

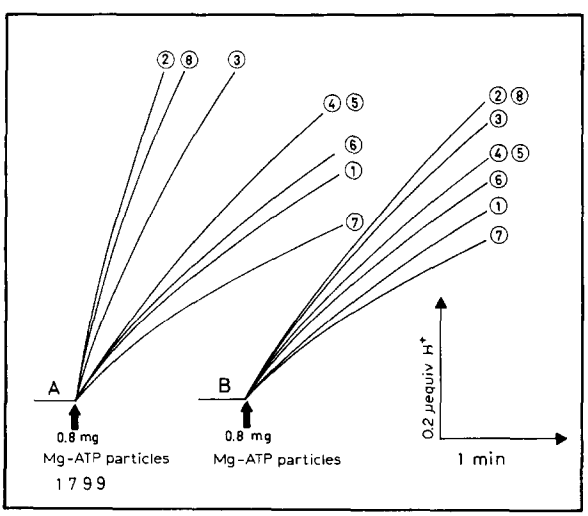


Fig. 5. Isolation of Mg–ATP particles with an activated ATPase activity. 4 mg of Mg–ATP particles were incubated in a mixture containing 250 mM sucrose, 4 mM potassium phosphate buffer (pH 7.0) and 0.5 mg/ml defatted bovine serum albumin. Further additions or omissions: Curves 1, 5 mM Tris–TES buffer (pH 7.0) instead of the phosphate buffer (control experiment); Curves 2, 5 mM succinate, 1 mM Mg^{2+}–ADP, 25 mM glucose, 25 I.U. of hexokinase; Curves 3, 5 mM succinate and 1 mM Mg^{2+}–ADP; Curves 4, as Curves 2 but *minus* succinate; Curves 5, as Curves 2 but *plus* 1.25 μg antimycin (before succinate); Curves 6, as Curves 2, but *plus* 15 μM 1799 (before succinate); Curves 7, the particles obtained as described under Curves 2 (2 mg) were re-incubated with 1.0 μg of inhibitor as described in Methods; Curves 8, as Curves 3 but *minus* Mg^{2+}–ADP. The particles were isolated from the media as described in Methods. Samples, containing 0.75 mg of protein were assayed for ATPase activity as described in Methods. This assay was carried out with the particles alone (B) and with the simultaneous introduction of 15 μM 1799 (A).

reached anaerobiosis the uncoupler-stimulated ATPase activity is the highest that can be obtained (Curve C1). The specific ATPase activity is comparable to that of the inhibitor-free AS particles (3–4 μequiv H^+/min per mg protein; compare Table I).

To prove that dissociation of the inhibitor from F_1 is indeed the reason for the greatly increased turnover capacity of F_1, Mg–ATP particles were centrifuged down as quickly as possible from different incubation media (Fig. 5). It is obvious that particles isolated from a medium that maintains State-3 conditions in the presence of an ATP-trapping system exhibit the highest uncoupler-induced ATPase activity (Curve A2). The ATPase activity in the absence of uncoupler is enhanced about 2-fold compared to the control (Curves B2 and B1).

From the ATPase activity of the particles obtained after different preincubations presented in Fig. 5, it can be concluded that for full activation of the uncoupler-stimulated ATPase activity, *i.e.* for a high degree of dissociation of the inhibitor

TABLE II

ACTIVATION OF THE UNCOUPLER-STIMULATED ATPase IN Mg–ATP PARTICLES INDUCED BY STATE-3 OXIDATION OF DIFFERENT SUBSTRATES

3.2 mg of Mg–ATP particles were incubated at 25 °C in a medium with a final volume of 5 ml containing 1.25 mmoles sucrose, 20 μmoles phosphate, 5 μmoles H_2O_2, 5 μg catalase, 5 μmoles Mg^{2+}–ADP, 125 μmoles glucose and 15 I.U. of hexokinase at a final pH of 7.0. Further additions were: A, 25 μmoles succinate; B, 7.5 μmoles NADH; C, 0.25 μmole TMPD and 25 μmoles ascorbate; D, none. 3.2 mg Mg–ATP particles were kept in 5 ml of 0.25 M sucrose *plus* 20 mM KCl. Re-isolation of the particles was performed as described in Methods. ATPase activities of 0.8 mg of particles were assayed essentially as described in Fig. 5. Expt 2 was done in the absence of bovine serum albumin. Rates of oxygen uptake of the particles during the pre-incubation in State 3 were measured in a parallel experiment. They were in: A, 130; B, 245; C, 40 natoms O/min per mg protein. Respiratory controls induced by Mg^{2+}–ADP were 1.6, 1.8, and 1.05, respectively.

Expt	*Substrate in preincubation*	*Addition of uncoupler (μM) at t= 1 min*		*ATPase activity (μequiv H^+/min per mg protein)*	
		1799	*FCCP*	*minus 1799*	*plus 1799*
1	—	—	—	0.15	0.20
	TMPD/ascorbate	—	—	0.19	0.25
	NADH	—	—	0.28	1.05
	Succinate	—	—	0.24	0.50
2	NADH	—	—	0.28	
		1	—	0.35	
		3	—	0.70	
		15	—	1.35	
		35	—	1.60	
		—	0.1	0.48	
		—	0.7	1.00	
		—	3	1.50	
		—	6	1.60	

from F_1, the following conditions must be fulfilled: coupled uninhibited electron transport is necessary; ATP formed during oxidative phosphorylation must be removed from the medium; Mg^{2+}–ADP, although capable of inducing high uncoupler-stimulated ATPase activity (see Fig. 4, Curve A4) is not required to induce dissociation of the inhibitor from F_1 (Fig. 5, Curve A8).

That the inhibitor and not some other component, indeed dissociates under State-3 conditions can be shown by re-incubating the particles as described under Curves 2 with an excess of inhibitor (Curves 7). Firstly, the ATPase activity is inhibited and secondly, an uncoupler cannot stimulate the ATPase activity further.

In the experiments described in Table II Mg–ATP particles were centrifuged under State-3 conditions in the presence of an ATP-trapping system but with other oxidizable substrates. It is clear that the uncoupler-stimulated ATPase activity is the highest with NADH as a substrate and the lowest with *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) *plus* ascorbate, presumably also because the particles are less coupled under these conditions.

Table II shows also that 6 μM 1799 or 0.6 μM FCCP give half-maximal stimulation of the ATPase activity. When the titration with uncoupler was done in the presence of bovine serum albumin (see Methods) the values were not much higher (7 and 0.7 μM, respectively). Comparable concentrations of 1799 and FCCP are necessary to give half-maximal stimulation of the ATPase activity of Mg–ATP particles, as described for Fig. 4C.

These values are high compared to those required for half-maximal stimulation of the ATPase activity in intact mitochondria, but in this case the absolute ATPase activity is higher too, in agreement with the assumption that the turnover number of the uncoupler remains constant (see also ref. 19).

DISCUSSION

The data reported in this paper support the view[8] that the inhibitor plays an important regulatory role in controlling the "back" flow of energy from ATP to energy-linked processes. Since the effect of the inhibitor on F_1 is non-competitive, the regulation takes the form of a control of the turnover capacity of F_1.

Most probably, the interaction of the inhibitor with F_1 proceeds via an intermediate state (see Fig. 6). First the inhibitor binds to the particles, presumably

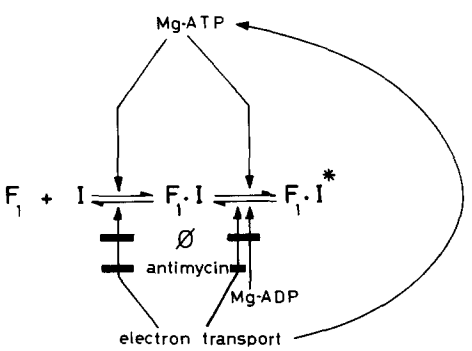


Fig. 6. Scheme of interaction of the ATPase inhibitor (I) with F_1. ϕ, uncoupler.

to F_1, without inhibiting it, followed by a transformation to the inhibited state, $F_1 \cdot I^*$. These transitions in the direction of the inhibited state are strongly promoted by Mg^{2+}–ATP and insensitive to the presence of uncoupler. Apparently only ATPase turnover conditions must be fullfilled. At least two pieces of evidence support the existence of this intermediate state: Firstly, A particles reconstituted with inhibitor to give partial inhibition are inhibited more strongly after incubation with Mg^{2+}–ATP. Secondly, activation of the uncoupler-stimulated ATPase activity by ADP occurs without appreciable dissociation of inhibitor from F_1.

The backward transition of the inhibited state to the uninhibited state is promoted by ADP or by coupled electron transport. That this effect of ADP is counteracted by an uncoupler is supported by the following lines of evidence: Firstly, incubation of A particles in the presence of uncoupler and Mg^{2+}–ATP gives the highest inhibition of the ATPase activity, indicating that the ADP formed in the preincubation is no longer effective in promoting the backward transition. Secondly, when uncoupler is added to Mg–ATP particles shortly after starting an ATPase reaction hardly any stimulation of the ATPase activity is observed, although ADP is formed progressively during the reaction. Also incubation of the particles in the presence of ADP *plus* uncoupler does not elicit a high ATPase activity.

We conclude that in coupled particles the reversible transition from the uninhibited to the inhibited complex of F_1 and inhibitor is regulated primarily by the ratio ATP/ADP. Furthermore, transition to the uninhibited state is strongly promoted by coupled electron transport. At the same time, dissociation of the uninhibited complex into free inhibitor and F_1 occurs under these conditions. The extent of dissociation seems to be dependent on the energy pressure exerted by the electron transport chain.

Conditions favouring transition to the uninhibited state or even dissociation of the inhibitor from the complex also strongly diminish the rate and extent of binding of an excess of added inhibitor to inhibitor-depleted particles or F_1. On the other hand, the dissociation of inhibitor from F_1 is counteracted by ATP formed during oxidative phosphorylation (Fig. 5, Curve A3). This could explain why succinate oxidation in recoupled AS particles does not diminish the interaction between excess inhibitor and the particles, while NADH oxidation does (Fig. 2, Curves 3 and 5). Apparently the energy pressure from the oxidation of succinate is not high enough in this case to overcome the "back" pressure by the Mg^{2+}–ATP.

It is worthwhile to emphasize here that the ATPase activity of untreated Mg–ATP particles is not stimulated to an appreciable extent by an uncoupler, because the rate-limiting step in the reaction is the turnover capacity of F_1. These particles are prepared by sonication of mitochondria under conditions where the transition of the F_1–inhibitor complex to the inhibited state is strongly favoured. Only when part of the inhibitor has been dissociated or when F_1 is transformed to the uninhibited state, the turnover capacity of F_1 increases so much that the energy-dissipating reactions become rate limiting for the ATPase activity. Energy dissipation can, of course, be stimulated strongly by an uncoupler, as is the case in intact mitochondria. Presumably the F_1–inhibitor complex in isolated intact mitochondria is partly in the uninhibited state. In experiments with intact mitochondria Bertina (unpublished observations) found that the uncoupler-stimulated

ATPase activity can be inhibited by about 80% by preincubation of the mitochondria with respiratory-chain inhibitors. He interpreted this result in terms of association of the inhibitor with F_1, as a consequence of the complete absence of electron transport.

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