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THE EQUILIBRIUM BETWEEN THE MITOCHONDRIAL ATPase (F_1) AND ITS NATURAL INHIBITOR IN SUBMITOCHONDRIAL PARTICLES

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SUMMARY

1. The reversible equilibrium between the mitochondrial ATPase (F_1) and its naturally occurring inhibitor in Mg–ATP submitochondrial particles has been studied under different conditions.

2. High ionic strength favours dissociation of the ATPase inhibitor as tested by ATPase and ATP-driven transhydrogenase activities.

3. Dissociation of the ATPase inhibitor results in an increased maximal velocity of the ATPase activity measured in the presence of uncoupler and an increased affinity for adenine nucleotides, in particular for ATP.

4. Association of the ATPase inhibitor with inhibitor-depleted Mg–ATP particles causes a slowing of the initial rate of succinate oxidation.

5. The antibiotic aurovertin stimulates the ATPase activity of Mg–ATP particles preincubated in the presence of a supply of oxidative energy. Bound aurovertin impedes the association of inhibitor-deficient particles with ATPase inhibitor.

6. The fluorescence of aurovertin bound to inhibitor-containing particles is much less than that of aurovertin bound to inhibitor-depleted particles.

7. The oligomycin-sensitivity-conferring protein, added either alone or in the presence or absence of membranous components of the ATPase complex, has little or no effect on the fluorescence of the F_1–aurovertin complex.

8. It is suggested that the ATPase inhibitor brings F_1 in a conformation denoted $*F_1$ that binds aurovertin with a low quantum yield, a decreased affinity and an increased binding capacity.

Abbreviations and symbols: F_1, mitochondrial ATPase; OSCP, oligomycin-sensitivity-conferring protein; I, mitochondrial ATPase inhibitor; A particles, submitochondrial particles prepared by sonication of beef-heart mitochondria in an alkaline solution at pH 9.2; AS particles, submitochondrial particles prepared by treatment of A particles with Sephadex G-50; Mg–ATP particles, submitochondrial particles prepared by sonication of beef-heart mitochondria in the presence of 15 mM $MgCl_2$ and 1 mM ATP (pH 7.5); TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; 1799, α,α′-bis-(hexafluoroacetonyl)acetone.

INTRODUCTION

Three substances are known that specifically interact with the soluble mitochondrial ATPase (F_1), a protein of molecular weight 360 000 [1] that has been isolated from beef heart and liver and purified to homogeneity [1–5].

(i) A naturally occurring ATPase inhibitor that has been isolated by Pullman and Monroy [6]. This is a soluble trypsin-sensitive protein of molecular weight 10 500 [4, 7] that strongly inhibits the ATPase activity of both isolated F_1 [6] and inhibitor-depleted AS particles [4]. Asami et al. [8] suggested that the ATPase inhibitor is an uni-directional inhibitor of F_1, i.e. that it inhibits all ATP-driven processes in submitochondrial particles, without affecting oxidative phosphorylation. We showed that the inhibition is of the non-competitive type and also reported the existence of a reversible equilibrium between the ATPase inhibitor and F_1, in vitro [9].

(ii) An oligomycin-sensitivity-conferring protein (OSCP), described and isolated in nearly pure form by MacLennan and Tzagoloff [10]. This is a soluble protein of molecular weight 18 000 [10], that not only combines with isolated F_1 [11], but also binds to the membranous part of the ATPase complex [10, 12]. OSCP is required for reconstitution of membrane vesicles with isolated F_1.

(iii) The antibiotic aurovertin, a potent inhibitor of oxidative phosphorylation [13, 14], but much less active in inhibiting ATP-driven processes catalysed by mitochondrial preparations [14–18]. Lardy and Lin [19] showed that the specific and stoichiometric binding (1 : 1) of the antibiotic to isolated F_1 is accompanied by at least a 50-fold enhancement of its fluorescence. The fluorescence properties of aurovertin bound to F_1 are not only strongly influenced by ligands of the enzyme [18–22] but also by the energy state of the mitochondrial membrane [20, 21]. These changes in the fluorescence of the antibiotic are generally interpreted as monitoring conformational changes of F_1, induced by ligands of the enzyme or by the membrane itself [18–23].

The purpose of this paper is to examine further the mutual interactions between the ATPase and these three substances, in order to gain further insight into the course of events taking place during energy conservation. Some of the results have been reported in an abstract [24].

METHODS AND MATERIALS

Preparations

Heavy beef-heart mitochondria [9], A particles [23], AS particles [9], Mg–ATP particles [9], coupling factor F_1 [9], the ATPase inhibitor [9] and OSCP [11] were prepared as described earlier. Aurovertin D was isolated in pure form in our laboratory as described by Bertina [18]. Physical data have been reported earlier [18, 22, 23].

Measurement of ATPase activity

Initial rates of ATPase activity were measured by the change of pH of the reaction medium with a sensitive pH-measuring system as described previously [9]. The standard reaction medium contained at 30 °C in a final volume of 1.5 ml: 375 μmoles sucrose, 3 μmoles ATP, 3 μmoles $MgCl_2$ and 7.5 μmoles potassium phos-

phate buffer at a final pH of 7.5, unless stated otherwise. All reactions were started by the addition of an appropriate sample of submitochondrial particles.

Measurements of oxygen consumption

Oxygen uptake was measured in a thermostatted vessel, equipped with a Teflon-covered Clark oxygen electrode (Yellow Springs Instr.) with an appropriate polarization circuitry. The reaction was started by the addition of 1 mg submitochondrial particles to a standard mixture at 30 °C containing in a final volume of 1.5 ml, 375 μmoles sucrose, 15 μmoles Tris–*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES) buffer, 1.5 μmoles $MgCl_2$ and 15 μmoles succinate, at a final pH of 6.7.

Measurement of the ATP-driven transhydrogenase [25]

Mg–ATP particles (0.6 mg protein), supplemented with 2 μg rotenone, were added to a reaction medium, maintained at 30 °C, containing, in a final volume of 2 ml, 500 μmoles sucrose, 150 μmoles ethanol, 5 μmoles $MgCl_2$, 0.3 μmole NADH, 0.8 μmole $NADP^+$, 100 μg alcohol dehydrogenase, 6 μmoles Tris–acetic acid buffer and the salts indicated in the figure, at a final pH of 8.0. After measuring the non-energy-linked transhydrogenase for 2 min, the energy-linked reaction was initiated by the addition of 5 μmoles ATP (pH 8). Formation of NADPH was followed spectrophotometrically at 340 nm. The determination of the rate of the energy-linked reaction required a small correction (5%) for the non-energy linked reaction.

Fluorimetric measurements

The fluorescence enhancement of aurovertin accompanying binding to F_1 or submitochondrial particles was recorded in a specially adapted Eppendorf fluorimeter, using the same filters as described earlier [23]. The standard medium contained, in a final volume of 1.5 ml, 375 μmoles sucrose, 37.5 μmoles glucose and 37.5 μmoles Tris–acetic acid buffer (pH 7.3). The fluorimeter was adjusted to the same sensitivity as used for experiments published before [23], by calibration with a standard solution of NADH.

Submitochondrial particle-ATPase inhibitor equilibrium

Association of the ATPase inhibitor with submitochondrial particles depleted of this protein was carried out essentially as described by Van de Stadt et al. [9]. The re-isolated particles were washed twice and suspended in 0.25 M sucrose. Dissociation of the ATPase inhibitor from Mg–ATP particles was achieved as follows (see ref. 9): Mg–ATP particles (3 mg protein) were incubated at 25 °C in 6 ml of a reaction mixture containing 250 mM sucrose, 10 mM Tris–acetic acid buffer (pH 7.3), 5 mM potassium phosphate buffer (pH 7.3), 1 mM $MgCl_2$, 1 mM ADP, 10 mM succinate (or 2 mM NADH), 25 mM glucose, 20 enzyme units hexokinase and 100 enzyme units catalase. After 3 min, 1 mM H_2O_2 was added and the suspension was immediately centrifuged at 200 000 × *g* for 10 min at room temperature. Re-isolated particles were washed twice and suspended in 0.25 M sucrose. These particles are termed "State-3 particles".

Analytical methods were the same as described previously [11, 23].

Materials

NAD^+, $NADP^+$, ATP, ADP, hexokinase, alcohol dehydrogenase and catalase were obtained from Boehringer und Söhne. Oligomycin was kindly provided by the Upjohn Chemical Co., the uncouplers α,α'-bis(hexafluoroacetonylacetone) (1799) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were a gift of Dr P. Heytler. TES was purchased from Calbiochem. All other chemicals were of analytical-grade purity. The enzymes were dialysed before use as described before [9, 23]. All organic acids used were neutralized with Tris to the desired pH.

RESULTS

The equilibrium between F_1 and its inhibitor during energy conservation

As shown by Van de Stadt et al. [9], dissociation of the endogenous ATPase inhibitor from tightly coupled Mg–ATP particles can be estimated by assaying the ATPase activity in the absence and presence of an uncoupler. When the inhibitor is associated with the particles, the turnover capacity of F_1 is the rate-limiting step in the ATPase activity. When the inhibitor is dissociated from the particles, the ATPase activity is related to the degree of coupling maintained by the particles, i.e. energy dissipation is the rate-limiting step. This latter process is strongly stimulated by an uncoupler and the rate of the ATPase activity is then proportional to the degree of dissociation of the ATPase inhibitor from Mg–ATP particles.

TABLE I

ACTIVATION OF THE ATPase ACTIVITY OF Mg–ATP PARTICLES

Mg–ATP particles (3 mg protein) were incubated in a standard medium containing, in a final volume of 6 ml, 250 mM sucrose, 10 mM Tris–acetic acid buffer (pH 7.3), 25 mM glucose and 100 units catalase. Further additions as indicated. (A) The initial ATPase activity of the isolated particles was tested in the absence and presence of 2 μM FCCP. (B) Following this assay, the isolated particles (2 mg protein) were re-incubated for 15 min in 1 ml medium at 25 °C containing 250 μmoles sucrose, 10 μmoles Tris–TES buffer (pH 6.6), 0.3 μmole $MgCl_2$ and 0.3 μmole ATP. In the experiment shown in the last line, 10 μg ATPase inhibitor was added to State-3 particles during the second incubation. After re-isolation the initial ATPase activities of the particles were assayed in the absence and presence of 2 μM FCCP.

Additions	Initial ATPase activity (μequiv H^+/min per mg protein)			
	(A)		(B)	
	+FCCP	−FCCP	+FCCP	−FCCP
None	0.14	0.13	0.07	0.06
KCl (50 mM)	1.03	0.62	0.83	0.40
Succinate (10 mM)	1.35	0.52	1.15	0.42
Succinate, KCl	1.93	1.20	1.80	0.92
Succinate, ADP (1 mM), phosphate (5 mM), $MgCl_2$ (1 mM), hexokinase (20 units)	2.85	0.65	2.40	0.55
ATPase inhibitor (10 μg)	—	—	0.10	0.10

Table I, Columns A, lists the initial ATPase activities, in the presence and absence of 2 μM FCCP, of Mg–ATP particles subjected to different treatments. It can be seen that high ionic strength (50 mM KCl) induces a partial dissociation of the ATPase inhibitor and a decrease of the degree of coupling of the particles. FCCP stimulates the ATPase activity by 65%. Succinate oxidation elicits a larger dissociation of the ATPase inhibitor. The ATPase activity is stimulated 150% by FCCP. Incubation under State-3 conditions produces the highest degree of dissociation of the ATPase inhibitor, while the particles remain relatively coupled, FCCP enhancing the ATPase activity 3.5-fold.

In order to test whether the ATPase inhibitor was dissociated from the particles by these treatments or remained bound to the particles in a non-inhibitory form [9], the isolated particles were incubated with Mg^{2+} and ATP after which the ATPase activity was again determined. This procedure has been shown to bring about association between F_1 and the inhibitor [4, 9]. Columns B show that incubation with Mg^{2+} and ATP caused little inhibition, unless ATPase inhibitor was also added.

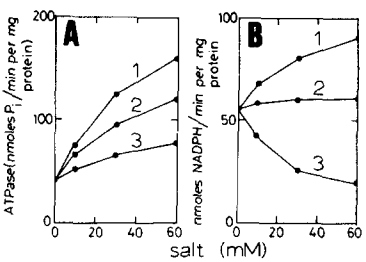


Fig. 1. Effect of salts on the ATPase activity and the ATP-driven transhydrogenase in Mg–ATP particles. (A) Mg–ATP particles (1 mg protein) were added to 1 ml reaction medium at 30 °C containing 250 μmoles sucrose, 3 μmoles Tris–acetic acid buffer, 2.5 μmoles $MgCl_2$ and 2.5 μmoles ATP. After 3 min the reaction was stopped by the addition of 1 ml 10 % (w/v) trichloroacetic acid to determine liberated inorganic phosphate as has been described previously [11]. Further additions were: 1, potassium acetate (pH 7.5); 2, KCl; 3, KNO_3. (B) The rate of the ATP-driven transhydrogenase was measured in Mg–ATP particles (see Methods and Materials) in the presence of: 1, potassium acetate (pH 8.0); 2, KCl; 3, KNO_3.

Fig. 1 summarizes results obtained earlier by Van de Stadt and Nieuwenhuis [26]. The ATPase activity (as measured by the liberation of phosphate over a 3 min period) of Mg–ATP particles suspended in a reaction medium of low ionic strength is stimulated most effectively by potassium acetate, while nitrate is less effective (Fig. 1A). Fig. 1B shows that the energy-linked transhydrogenase reaction [25] driven by ATP is stimulated by acetate and inhibited by nitrate, while chloride takes an intermediate position. To understand these results, the effect of salts was studied on the ATPase activity of submitochondrial particles containing various amounts of the ATPase inhibitor.

In Table II, the initial ATPase activities, as measured with a sensitive pH electrode, are listed of A particles (endogenously uncoupled particles, deficient in ATPase inhibitor), Mg–ATP particles and Mg–ATP particles depleted of ATPase inhibitor by centrifugation under State-3 conditions (denoted State-3 particles; see Methods and Materials).

TABLE II

ACTIVATION BY SALTS OF THE ATPase ACTIVITY OF Mg–ATP PARTICLES

Initial ATPase activities were measured of appropriate samples of A particles, Mg–ATP particles and State-3 particles (NADH, see Methods and Materials) in the presence of valinomycin, nigericin and FCCP.

Expt	Additions				Initial ATPase activity (μequiv H^+/min per mg protein)		
	KCl (mM)	Valinomycin (μg/mg of protein)	Nigericin (μg/mg of protein)	FCCP (μM)	A particles	Mg–ATP particles	State-3 particles
1	–	–	–	–	6.5	0.13	0.34
	15	–	–	–	6.3	0.21	0.39
	50	–	–	–	5.4	0.26	0.44
2	50	0.2	–	–	–	0.26	0.50
	50	0.2	0.2	–	–	0.29	3.50
3	–	–	–	1.4	6.7	0.15	3.50
	–	–	–	3.2	–	–	4.50

Expt 1 (see Table II) shows that 50 mM KCl slightly inhibits the initial ATPase activity of A particles, stimulates that of Mg–ATP particles by 100%, and that of State-3 particles by 30%. More important is that the rate of the ATPase activity of Mg–ATP particles declines in time after addition of the particles to the reaction medium, this effect being less pronounced with higher concentrations of KCl (not shown). The decline in activity of State-3 or A particles was much less, no more, in fact, than to be expected from the competitive inhibition by ADP formed during the reaction. This suggests that some of the ATPase inhibitor is bound to the original Mg–ATP particles in a non-inhibitory form [9] and becomes rapidly associated in an inhibitory form upon addition of the particles to the ATPase assay mixture, this process being prevented by high ionic strength (cf. Table I, Rows 1 and 2).

Expt 2 of Table II shows that State-3 particles can be effectively uncoupled

TABLE III

KINETIC PARAMETERS OF THE ATPase ACTIVITY

The concentrations of $MgCl_2$–ATP (pH 7.5) ranged from 0.2 to 10 mM. Incubations were done in the absence and presence of 0.25 mM $MgCl_2$–ADP (pH 7.5). The initial velocities were plotted in a double-reciprocal plot versus the concentration of ATP. The resulting kinetic parameters are given.

Expt	Particles	K_m (ATP) (μM)	K_i (ADP) (μM)	v (μequiv H^+/min per mg protein)
1	Mg–ATP particles (0.5 mg)	185	139	0.16
2	Mg–ATP particles (0.5 mg), FCCP (2 μM)	164	120	0.16
3	State-3 particles (0.25 mg)	193	132	0.70
4	State-3 particles (0.25 mg), FCCP (2 μM)	50	70	4.10

by 50 mM KCl in the presence of valinomycin and nigericin. The extent of uncoupling is comparable to that induced by 1.4 μM FCCP (Expt 3).

It has been shown previously that interaction of the ATPase inhibitor with AS particles results in non-competitive inhibition [9]. The kinetic parameters of the ATPase activity of Mg–ATP particles listed in Table III (Expts 1 and 3) lead to the same conclusion. It is also shown that an uncoupler hardly affects these parameters when the inhibitor is associated with the particles (Expt 2). In inhibitor-deficient State-3 particles (see Methods and Materials), however, 2 μM FCCP not only enhances the maximal velocity strongly, but also increases the affinities for adenine nucleotides, in particular for ATP (Expt 4).

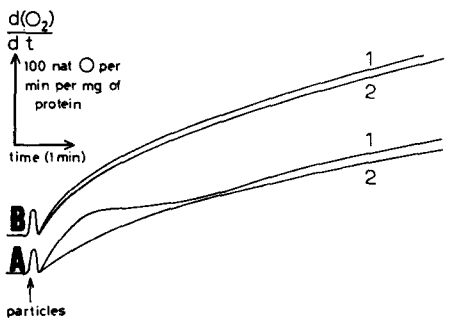


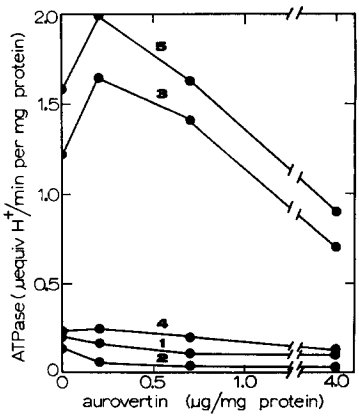
Fig. 2. Inhibition of the initial rate of oxygen uptake by the ATPase inhibitor. State-3 particles (NADH, see Methods and Materials) were incubated with $MgCl_2$ and ATP, as described in Table I, in the absence (Curves 1) and presence of ATPase inhibitor (Curves 2). After re-isolation of the particles, the rate of oxygen consumption was recorded with succinate as the substrate; A, standard medium (see Methods and Materials); B, standard medium plus 5 mM phosphate buffer (pH 6.7) and 1 mM ADP.

The ATPase inhibitor dissociates from Mg–ATP particles when coupled electron transport is initiated, this process being most rapid under State-3 conditions [9]. To test whether the ATPase inhibitor has an effect on the oxidation velocity, inhibitor-deficient State-3 particles were incubated with $MgCl_2$ and ATP, with or without the ATPase inhibitor. As shown in Fig. 2 succinate oxidation sets in more rapidly in particles depleted of the ATPase inhibitor than in particles containing the inhibitor (Curves A). The traces in Fig. 2 suggest that about 2 min is required for the dissociation of sufficient inhibitor to give an oxidation rate similar to that of the inhibitor-deficient particles. The assay was carried out at a low pH (6.7) to slow down dissociation of the inhibitor [4]. Under State-3 conditions (Curves B) little difference between the two particles is found, suggesting that dissociation of the inhibitor is too rapid under these conditions to be detected with this method. This explains why the ATPase inhibitor does not inhibit oxidative phosphorylation [6, 8]. No difference in the initial rate of NADH oxidation was found between the two types of particles. The initial rate of oxygen consumption was, however, about five times that for succinate. This may be because the high energy pressure generated by the high oxidation rate of NADH causes the dissociation to be too rapid to be detected by this method.

Effect of aurovertin on the ATPase activity of Mg–ATP particles

In the experiment described in Fig. 3, Mg–ATP particles were preincubated

with the indicated amounts of aurovertin under conditions described in the legend. After 1 min preincubation, a small aliquot of the suspension was immediately added to an ATPase assay mixture (see Methods and Materials). The initial rates of the ATPase activity, measured in the absence and presence of 2 μM FCCP, are plotted versus the aurovertin concentration. It can be seen that during succinate oxidation (Curves 3 and 5) preincubation with small amounts of aurovertin (0.2 μg/mg protein), sufficient to inhibit oxidative phosphorylation, brought about an increase of the uncoupler-stimulated ATPase activity (cf. Curve 4). Much higher concentrations of aurovertin are necessary to inhibit the ATPase activity [14–18]. Preincubation in the standard medium without succinate resulted in a slight and progressive inhibition by aurovertin (Curve 1). Interestingly, preincubation in the presence of ATP caused a 50 % inhibition of the ATPase activity by 0.2 μg aurovertin per mg protein (Curve 2).



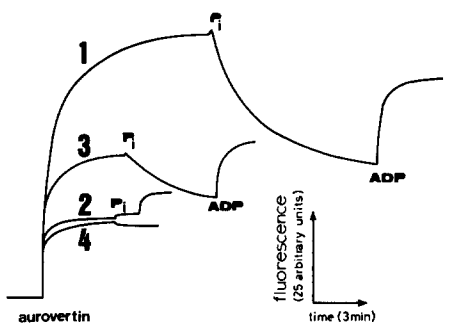


Fig. 3. Effect of aurovertin on the ATPase activity in Mg–ATP particles. Mg–ATP particles (2 mg protein) were preincubated for 1 min at 30 °C in a reaction medium containing, in a final volume of 2 ml 500 μmoles sucrose, 10 μmoles Tris–acetic acid buffer (pH 7.3), 0.8 μmole $MgCl_2$, 25 units catalase and the amounts of aurovertin indicated in the figure. Further additions: Curve 1, none; Curve 2, 0.8 μmole ATP; Curves 3 and 4, 10 μmoles succinate and 6 μmoles phosphate buffer (pH 7.3); Curve 5, 10 μmoles succinate, 6 μmoles phosphate buffer (pH 7.3) and 0.8 μmole ADP. If succinate was present 1 μmole H_2O_2 was added after 30 s. After the preincubation the initial ATPase activity was assayed by introducing an appropriate sample of the preincubation mixture into a reaction mixture as described in the Methods and Materials. Curves 3 and 5 were measured with 2 μM FCCP in the assay mixture. Under the conditions of Curves 1 and 2, the addition of 2 μM FCCP made no difference.

Fig. 4. The effect of the ATPase inhibitor on the fluorescence of aurovertin bound to different submitochondrial particles. 200 nM aurovertin was added to particles (1 mg protein), suspended in 1.5 ml standard medium. Further additions: 5 mM potassium phosphate buffer (pH 7.3) and 1 mM ADP. Curve 1, AS particles; Curve 2, AS particles reconstituted to the extent of 95 % with ATPase inhibitor; Curve 3, State-3 particles (succinate, see Methods and Materials); Curve 4, Mg–ATP particles.

Effect of the ATPase inhibitor and OSCP on the fluorescence of aurovertin bound to F_1

In Fig. 4, Trace 1, the aurovertin fluorescence enhancement is recorded for inhibitor-depleted AS particles. The extensive and slow quenching of the fluorescence by phosphate is partly reversed by ADP. The effects of ligands of F_1 on the

fluorescence of the complex between aurovertin and AS particles have been discussed in detail [23]. Trace 2 of Fig. 4 shows that nearly complete association of AS particles with ATPase inhibitor [23] causes a strong quenching of the aurovertin fluorescence. Phosphate does not further quench the fluorescence, and ADP produces only a relatively small enhancement. Analysis of the kinetics of the fluorescence enhancement in Trace 2 reveals only two fast phases, an initial jump in the fluorescence followed by a fast phase (rate constant $2.6 \cdot 10^{-2}\ s^{-1}$). A slow phase in the fluorescence enhancement in particles free from inhibitor [23], interpreted as a conformational change in F_1 induced by aurovertin [22], was not found with inhibitor-containing particles. Trace 3 of Fig. 4 shows that the fluorescence of the complex between aurovertin and State-3 particles, which are partially deficient in inhibitor, is considerably higher than that of Mg–ATP particles (Trace 4).

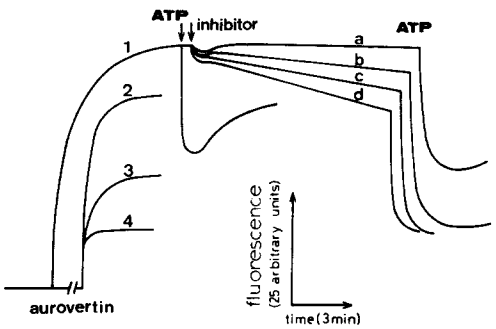


Fig. 5. Effect of the ATPase inhibitor on the fluorescence of the particle–aurovertin complex. 200 nM aurovertin was added to A particles (0.75 mg protein), suspended in 1.5 ml medium containing 375 μmoles sucrose, 22.5 μmoles Tris–TES buffer (pH 6.6) and 0.6 μmole $MgCl_2$. Further additions: Curve 1, 0.2 μmole ATP or ATPase inhibitor; a, 0.6 μg; b, 1.5 μg; c, 4.5 μg; d, 12 μg; Curve 2, 0.2 μmole ATP added 5 min before aurovertin; Curve 3, 4.5 μg ATPase inhibitor added 5 min before aurovertin; Curve 4, 4.5 μg ATPase inhibitor and 0.2 μmole ATP added 5 min before aurovertin.

Trace 1 of Fig. 5 illustrates that even an excess of ATPase inhibitor only slowly quenches the fluorescence of the aurovertin–A particle complex, suspended at a low pH (6.6). Subsequent addition of a small concentration ATP (0.13 mM), however, induces a rapid quenching, the extent being considerably larger in the presence than in the absence of the ATPase inhibitor. Trace 3 shows that a 5 min preincubation of A particles with 6.0 μg ATPase inhibitor per mg particle protein causes considerable association in the absence of ATP (see also ref. 9). The comparatively slow rate of quenching in Trace 1c suggests that bound aurovertin impedes the association of the ATPase inhibitor (cf. Trace 3). In the presence of ATP, however, association seems to be completed within 1 min.

The effect of adding OSCP on the fluorescence of aurovertin bound to isolated F_1 was also tested. An excess of OSCP (16 μg) did not have measurable effects on the fluorescence of a complex between 200 nM aurovertin and 75 μg F_1, suspended in 1.50 ml 25 mM Tris–acetate buffer (pH 7.5). Preincubation of F_1 and OSCP did not affect the final aurovertin fluorescence level as compared with that obtained with F_1 alone. The quenching found on addition of an insoluble membrane preparation depleted of F_1 and OSCP (oligomycin-sensitive ATPase extracted with NaBr and

NH_4OH, [10]) to the complex of F_1 and aurovertin in the presence of OSCP could be completely accounted for by the light scattering. It cannot be excluded, however, that a conformational change in F_1 is induced by OSCP, that is not expressed in a change of the aurovertin fluorescence.

Binding of aurovertin to AS particles reconstituted with ATPase inhibitor

Binding parameters of aurovertin to AS particles associated to the extent of 85% with ATPase inhibitor (estimated from specific ATPase activities) were obtained by measurement of the fluorescence of bound aurovertin (the AS particles used in these measurements originated from the same batch of particles as used before [23]). Fig. 6A shows the titration of particles with aurovertin in a standard medium. Fig. 6B is a double-reciprocal plot of the final fluorescence yield (ΔF) versus the protein concentration at different concentrations of the fluorochrome. By extrapolation to infinite protein concentration, the fluorescence of the completely bound aurovertin may be calculated. Fig. 6C, the plot of the fluorescence at infinite protein concentration versus aurovertin concentration, yields the relative quantum yield. This value was used to construct the Scatchard [27] plot shown in Fig. 6D from the titration data in Fig. 6A. Binding parameters calculated from Scatchard plots under various conditions are listed in Table IV. It can be seen that under all conditions tested, there

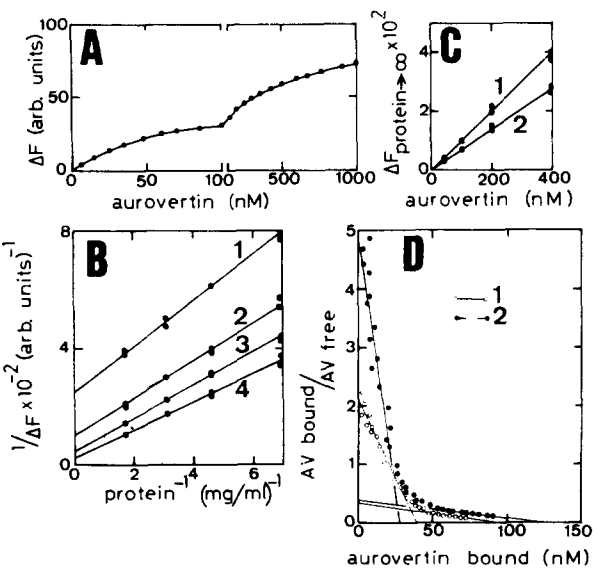


Fig. 6. Binding of aurovertin to AS particles reconstituted with ATPase inhibitor. AS particles were reconstituted with the ATPase inhibitor to the extent of 85% (see Methods and Materials). (A) Titration of particles (0.32 mg protein/ml), suspended in the standard medium, with aurovertin. Final fluorescence levels were recorded in separate experiments. (B) Double-reciprocal plot of the final fluorescence versus the protein concentration. Concentrations of aurovertin (nM): Curve 1, 41; Curve 2, 100; Curve 3, 200; Curve 4, 400. (C) Plot of the fluorescence yield extrapolated to infinite protein concentration versus the aurovertin concentration; Curve 1, standard medium; Curve 2, in the presence of 1 mM $MgCl_2$ and 5 mM phosphate buffer (pH 7.3). (D) Scatchard plot [27], derived from the titration curve A and the relative quantum yield; Curve 1, standard medium; Curve 2, in the presence of 1 mM $MgCl_2$ and 5 mM phosphate buffer (pH 7.3). AV, aurovertin.

TABLE IV

BINDING PARAMETERS OF AUROVERTIN TO AS PARTICLES RECONSTITUTED WITH ATPase INHIBITOR

Binding data were obtained essentially as described in Fig. 6. The ligands tested were added before aurovertin, with the exception of ATP, which was added after the aurovertin fluorescence had reached a final level. n_1 stands for the concentration of strong binding sites, n_2 for the concentration of weaker binding sites, K_{D_1} and K_{D_2} for the dissociation constants of these sites and Q_{rel} for the relative quantum yield.

Additions	Q_{rel} (units/nM aurovertin)	n_1 (nmole/mg of protein)	n_2 (nmole/mg of protein)	K_{D_1} (nM)	K_{D_2} (nM)
None	1.0	0.11	0.16	18	260
$MgCl_2$ (1 mM)	0.8	0.11	0.23	20	255
Phosphate (5 mM)	0.8	0.10	0.25	10	260
$MgCl_2$, phosphate	0.7	0.09	0.28	5	310
ADP (1 mM)	1.0	0.10	0.21	10	280
$MgCl_2$, phosphate, ADP, hexokinase (5 units)	0.8	0.09	0.38	4	260
$MgCl_2$, phosphate, ATP	0.7	0.09	0.37	15	300
ATP (1 mM)	0.7	0.09	0.35	12	290

are two aurovertin-binding sites, one with high affinity (concentration, n_1) and the other with lower affinity (concentration, n_2).

Quenching of the aurovertin fluorescence by the ATPase inhibitor is mainly caused by a decrease in the relative quantum yield. Aurovertin binding data to inhibitor-deficient AS particles under exactly the same conditions as in Table IV (no additions) yielded the values: $Q_{rel} = 2.3$, $n_1 = 0.20$, $n_2 = 0.05$, $K_{D_1} = 26$ and $K_{D_2} = 100$ ([23], Table III). Furthermore, the concentration of weak binding sites (n_2) is increased by binding of ATPase inhibitor, at the expense of the number of strong binding sites, and the affinity of the weak binding sites is considerably lowered.

The effect of ligands of the ATPase on the binding parameters of inhibitor-containing particles is much smaller than in case of AS particles (cf. Table III, [23]). The addition of phosphate or ATP, with or without $MgCl_2$, has no effect on the concentration of strong binding sites but the affinity of aurovertin for these sites is increased. The concentration of weak binding sites is increased, without any effect on their affinity. ATP or phosphate reduce the quantum yield by only 0.2–0.3 unit, compared with 1.5 units in AS particles [23].

Similar results were obtained with A particles re-associated with ATPase inhibitor to the extent of 95%. In this case the concentration of strong binding sites was even smaller (0.06–0.07 nmole/mg of protein).

DISCUSSION

Dissociation of the ATPase inhibitor from Mg–ATP particles is brought about by the supply of oxidative energy, especially under State-3 conditions. A high ionic strength also induces a partial dissociation (Table I). The effects of salts on ATP-

driven processes in Mg–ATP particles illustrate the regulating role of the ATPase inhibitor in energy conservation. The results of Table II indicate that the stimulation of the ATPase activity by salts (Fig. 1A) is caused by a partial dissociation of bound inhibitor. The different effectiveness of the anions in stimulating the ATPase activity can be attributed mainly to their capacity to enhance the maximal velocity of the ATPase [28]. Acetate gave the highest value whereas the most chaotropic anion tested here, nitrate, gave the lowest. The latter anion is the most effective inactivator of F_1, presumably by dissociating F_1 into subunits [2, 29].

The effect of salts on the ATP-driven transhydrogenase (Fig. 1B) can be explained by a balance between two effects: partial dissociation of the ATPase inhibitor causing an increased turnover of the ATPase, and inactivation of the ATPase enzyme by salts like nitrate. High concentrations of salts also considerably decrease the P/O ratio with Mg–ATP particles [30].

The inhibitory effect of the ATPase inhibitor (Fig. 2) could only be demonstrated under conditions where the rate of dissociation of the inhibitor is small, i.e. at a low pH, a low rate of oxygen consumption and in the absence of ADP and phosphate. This suggests that the inhibitor has to dissociate before energy generated by the respiratory chain can flow into F_1.

Association of AS particles with the ATPase inhibitor strongly quenches the fluorescence of bound aurovertin (Fig. 4) and reduces the effects of ligands like phosphate, ATP and ADP. Similar observations were made with isolated inhibitor-free F_1 (Van de Stadt, R. J., unpublished experiments).

The preceding paper [23] describes the effects of ligands on the fluorescence of the complex between aurovertin and inhibitor-depleted AS particles. To explain the results we proposed that there are two conformations of F_1 in equilibrium with each other, termed F_1 and $*F_1$; F_1 binds aurovertin in a highly fluorescent form, while $*F_1$ binds aurovertin in a less fluorescent form. The equilibrium is influenced by binding of ligands. ATP and phosphate induce a shift to conformation $*F_1$; ADP shifts the equilibrium to conformation F_1.

The results of Table III suggest that reconstitution of AS particles with the ATPase inhibitor induces conformation $*F_1$. This conclusion is further supported by the fact that reconstitution of AS particles with inhibitor prevents a slow phase in the aurovertin-fluorescence enhancement.

It has been discussed previously [23] that this slow phase in the fluorescence rise, upon addition of aurovertin to AS particles, may represent a conformational change of the enzyme, induced by aurovertin, giving rise to a higher quantum yield of bound aurovertin. The experiments shown in Fig. 3 indicate that low concentrations of aurovertin, if preincubated under conditions in which oxidative energy is supplied, stimulate the ATPase activity of Mg–ATP particles. The most probable explanation is that aurovertin facilitates dissociation of the ATPase inhibitor induced by electron transport, since it tends to induce conformation F_1 which is unfavourable for binding of the ATPase inhibitor. In line with this interpretation, bound aurovertin impedes the association of A particles with an excess of ATPase inhibitor (see Fig. 5).

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