EFFECT OF PREINCUBATION WITH ATP ON ATP-DEPENDENT REACTIONS IN SUB-MITOCHONDRIAL PARTICLES

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1. Introduction

During the course of our investigations on the mechanism of uncoupling, we observed that the binding of uncoupler to mitochondria and submitochondrial particles was considerably inhibited by ATP (unpublished). Since protein subunits of the mitochondrial ATPase complex seem to be involved in uncoupler binding [1,2] it was of interest to study the effect of preincubation with ATP on membrane energization using energy-linked reactions as the criteria. The results presented here clearly indicate that pre-treatment with ATP is deleterious to ATP-dependent membrane energization and that during preincubation with ATP, proteins are released from the particles.

2. Materials and methods

Bovine heart submitochondrial AE-particles* [3] were suspended (1 mg protein/ml) in 5 ml of a medium containing 100 mM Tris–SO_4 buffer, pH 7.5, 30 mM $MgCl_2$, 1.5 mM EDTA and varying concentrations of ATP as indicated. After incubation at 0–2°C for 30 min, the particles were sedimented by centrifugation at 105 000 × *g* in a Beckman Model L-2 ultracentrifuge, washed once with 5 ml of 10 mM Tris–SO_4 buffer (pH 7.5) containing 5 mg BSA/ml and sedimented as before. The washed particles were resuspended in 1 ml of 0.25 M sucrose containing 10 mM Tris–SO_4 buffer, pH 7.5 and used for assays.

ATPase activity was determined essentially as described by Veldsema-Currie and Slater [4]. ATP-dependent succinate-linked reduction of NAD^+ by reversed electron transport was determined with the addition of saturating concentrations of coupling factor B [5]. In the reaction system EDTA (1.5 mM) replaced DTT. Under these conditions about 80% of maximal activity (with DTT added) was obtained. Succinate-mediated reduction of NAD^+ by reversed electron transport driven by energy derived from the oxidation of ascorbate–TMPD in the presence of antimycin and oligomycin was determined as described by Kurup and Sanadi [6]. ATP–P_i exchange activity was assayed with the addition of Factor B [7]. Orthophosphate was determined by the method of Fiske and Subba Row [8]. The experiments to test the release of proteins during preincubation were carried out as follows: submitochondrial AE-particles (30–50 mg protein) were washed twice with 5 ml of the binding medium, the washed particles were suspended in 0.25 M sucrose and preincubation carried out as described earlier. The particles were sedimented by centrifugation and the supernatant solution was treated five times with about 1 g portions of Dowex

*Abbreviations: AE-particles, bovine heart submitochondrial particles prepared by treatment with NH_4OH and EDTA, at pH 8.8; DTT, dithiothreitol; TMPD, *N,N,N'N'*-tetramethyl-*p*-phenylenediamine; SDS, sodium dodecylsulfate

1-C1 (Biorad AG1-X2) [9] to remove nucleotides which seemed to interfere with subsequent electrophoresis. After the final centrifugation, the solution was lyophilized, taken in SDS (4%, 0.8 ml) and mercaptoethanol (0.2 ml, 10%), boiled and electrophoresed (0.2 ml) on polyacrylamide gels (10% acrylamide) essentially as described by Weber and Osborn [10]. The gels were stained with Coomassie Blue and scanned at 600 nm in a Gilford Model 240 spectrophotometer with Model 2410 Linear Transport attachment. For molecular weight determination, ovalbumin and cytochrome *c* were used as internal standards.

3. Results

3.1. *Energy-linked reactions*

On pretreatment with ATP, AE-particles showed significant loss of ATP–P_i exchange and reversed electron transport activity energized by ATP but not by substrate oxidation (table 1). In the absence of added Factor B, ATP-dependent NAD^+ reduction in AE-particles is stimulated by low concentrations of oligomycin [3]. Preincubation with ATP decreased by more than 50% the oligomycin-stimulated activity as well. In these particles, NAD^+ reduction driven by aerobic energy is stimulated greatly by oligomycin [6]. Particles preincubated with ATP still required oligomycin for optimal activity.

3.2. *ATP hydrolysis*

During preincubation of the particles with ATP, which was carried out in this experiment at 38°C because the release of P_i showed better time dependence, there was considerable ATP hydrolysis. The extent of ATP hydrolysis was dependent on the concentration of ATP in the incubation medium (fig.1). However, when the particles were separated from the

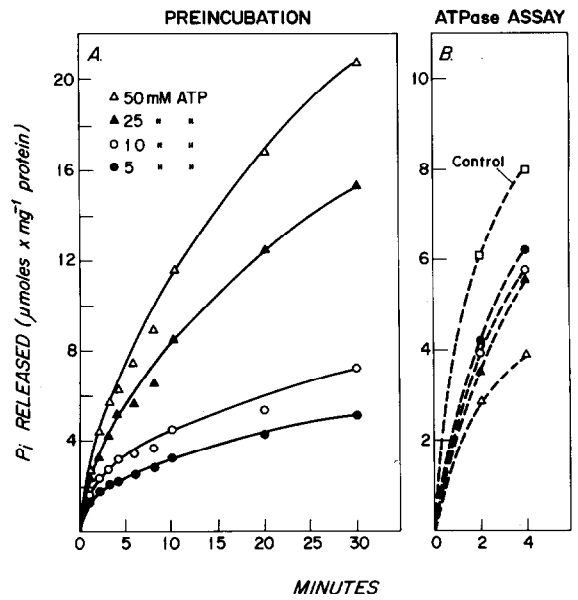

Fig.1. Effect of preincubation with ATP on the ATPase activity of AE-particles. Effect of ATP concentration on hydrolysis during preincubation is shown in fig.1A. Samples (0.5 ml) were withdrawn at the time periods indicated and P_i estimated. fig.1B represents the effect of preincubation on the ATPase activity of AE-particles. The particles were incubated at 0–2°C with and without addition of ATP for 30 min, sedimented, washed and the ATPase activity assayed with 12.5 mM ATP in the assay medium in all cases [4].

Table 1
Effect of preincubation with ATP on energy-linked reactions in AE-particles

ATP in pre-incubation medium (mM)	ATP–P_i exchange	ATP-dependent NAD^+ reduction	Oxidation-dependent NAD^+ reduction
0	100.0	50.0	50.0
5	50.4	37.5	45.0
10	42.0	35.0	47.5
25	18.3	32.5	52.5
50	12.8	20.5	70.0

Particles were incubated with or without ATP for 30 min at 0–2°C, washed and assayed. Activity is expressed as nmol/min/mg particle protein. The results in the reversed electron flow assay were similar when 0.5 mM DTT was used instead of ATP.

Table 2
Effect of Mg^{2+} ion concentration in the preincubation medium on 'ATP effect'

Preincubation medium			ATPase	ATP-linked NAD^+ reduction
Mg^{2+} Ions (mM)	ATP (mM)	P_i released during preincubation (μmol/mg/30 min)	(μmol P_i/min/mg)	(nmol/min/mg)
0	0	–	1.42	40
0	10	0.19	1.28	35
0	50	0.79	1.08	30
30	0	–	1.27	42
30	50	1.57	0.68	18
60	0	–	1.47	40
60	10	0.80	0.83	27
60	50	1.51	0.47	12

Particles (1 mg/ml) were incubated at 0–2°C for 30 min in preincubation medium containing Mg^{2+} ions and ATP as indicated, sedimented and washed. ATPase and reversed electron transport activities were determined as in table 1. The preincubation medium was analyzed for P_i after sedimentation of the particles.

preincubation medium, their ATPase activity was decreased, the extent of decrease depending on the concentration of ATP in the preincubation medium (fig.1B). The ATPase activity was lost to the extent of about 50% on preincubation with 50 mM ATP. The results presented in table 2 indicate that the presence of Mg^{2+} ions in the preincubation medium is necessary for the 'ATP effect'.

We attempted to determine whether ATPase turnover was essential to produce the 'ATP effect' by incubating with ATP in the presence of oligomycin but failed since bound oligomycin was not removed even after repeated washed of the particles with BSA.

Preincubation with ADP (50 mM) did not affect the energy-linked functions in the particle. However, the ATPase activity of the particles was decreased by 40% on ADP treatment. Preincubation with succinate (10,25, or 50 mM) had no effect and ATP exerted its effect even when preincubated in the presence of succinate.

When preincubation was carried out in the presence of CTP (50 mM) instead of ATP, the P_i liberated was one-fifth of that from ATP (0.45 mol P_i liberated/mg particle protein in 30 min as compared to 2.6 mol P_i liberated from ATP) and the particles did not show any loss in activity. Sub-mitochondrial particles hydrolyze CTP at a much slower rate than they do ATP [11].

3.3. *Protection by uncouplers*

It was observed that if the uncoupler, 2-azido-4-nitrophenol, was present in the preincubation medium, the 'ATP effect' was decreased to some extent, particularly when the ATP concentrations were low (table 3). Dinitrophenol (1 mM) was only 20% effective as NPA.

3.4. *Partial reversal by mitochondrial ATPase*

Addition of ATPase to the particles after ATP treatment increased the reversed electron transport activity. For example, ATP-treatment lowered the reversed electron transport activity of the particles from 28.9 to 8.9 (nmol NAD^+ reduced/min/mg protein). On addition of ATPase (40 μg/mg particle protein), the activity was increased to 16.7. Further additions led to inhibition of activity. Also, ATPase had no effect when added after the reaction was initiated. Since AE-particles are not significantly deficient in ATPase, this may indicate that during preincubation with ATP, the ATPase suffers some damage.

3.5. *Protein release*

Experiments were carried out as described under Materials and methods to see whether any protein was

Table 3
Prevention of the 'ATP effect' by azidonitrophenol

Preincubation medium		ATPase	ATP-linked NAD^+ reduction
ATP (mM)	Azidonitro-phenol (mM)	μmol P_i/min/mg	nmol/min/mg particle protein
0	0	0.85	38.9
10	0	0.51	21.1
10	1	0.66	33.3
25	0	–	16.6
25	1	–	21.1
50	0	0.22	5.6
50	1	–	14.4

AE-particles were preincubated with ATP and uncoupler as indicated, washed, resuspended in sucrose and the ATPase (30°C) and reversed electron transport activity (38°C) determined. Details are given in the Materials and methods section. Reversed electron transport was determined in the presence of factor B.

released during the preincubation with ATP. Some typical scans of the SDS gels are shown in fig.2. The results indicate the ATP-dependent release into the medium of proteins of approximate subunit mol. wt 45 000 and 65 000. The protein release is largely inhibited by oligomycin. In the absence of ATP, no protein was detected. Another protein with subunit molecular weight equal to that of cytochrome *c* is also seen in the scan. The release of this protein occurs even in the presence of oligomycin.

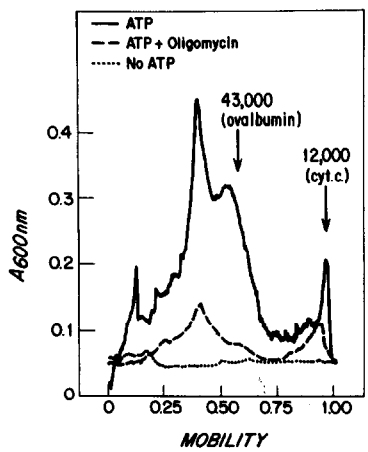

Fig.2. Polyacrylamide gel electrophoresis of proteins released during preincubation. Washed AE-particles (48 mg protein) were incubated with the addition of no ATP (- - -), 50 mM ATP (——) or 50 mM ATP + 200 μg oligomycin (. . . .). The supernatant was processed for gel electrophoresis as described in the Materials and methods section. The arrows indicate the mobility of internal standards.

4. Discussion

The results presented in this paper give ample indication that ATP-induced energization leads to inactivation of the ATPase system and associated utilization of ATP for energy-dependent reactions. This inactivation does not affect the utilization of energy generated by aerobic oxidation. Our results also provide good indication that proteins are released from the particles during preincubation with ATP, although further work is needed to establish the relationship between the protein release and the loss of activity. If one assumes that protein release is more than a coincidence, then the possibilities are that the proteins are part of the ATPase system, or that they are regulatory components governing enzyme activity and energy transduction. The molecular weights of the subunits (45 000 and 65 000) are distinct from those of the ATPase system [11]. These results may bear on the regulation of ATP production within mitochondria. Earlier work has shown [12] that a medium of high ionic strength and energization promote the dissociation of the ATPase inhibitor. It is unlikely that the 'ATP effect' is brought about by such a mechanism since we find a decrease in ATPase activity

and not an increase. It is becoming increasingly evident that ATPase activity is modulated by nucleotides bound to the catalytic and regulatory sites on the enzyme [13,14]. The relationship of the present findings to this type of regulation needs further examination.

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