Biochimica et Biophysica Acta, 462 (1977) 323–332

BBA 47383

COLD LABILITY OF MEMBRANE-BOUND F_1-ATPase

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(Received March 8th, 1977)

SUMMARY

1. Preincubation of MgATP submitochondrial particles with EDTA or Tris · HCl liberated a measurable amount of ATPase inhibitor that could be rapidly purified using only trichloroacetic acid precipitation and heat treatment.

2. In spite of the emergence of high ATPase activity, a considerable amount of ATPase inhibitor was left in the particles. Comparative analysis of other submitochondrial preparations indicated that only AS-particles were effectively depleted.

3. The high ATPase activity of inhibitor-deficient particles, was labile at low temperature provided that the exposure to cold was done in the presence of MgATP. Other nucleotides could not substitute for ATP. Glycerol inhibited and salts enhanced the cold inactivation of membrane-bound F_1-ATPase. Isolation of F_1-ATPase from cold-inactivated particles yielded a soluble preparation of correspondingly lower activity.

4. It is concluded that together with the increase of ATPase activity, the ATP-dependent cold lability of membrane-bound F_1-ATPase and the dislocation of ATPase inhibitor at non operative sites reveal the extent of ATPase complex disorganization.

INTRODUCTION

The mitochondrial ATPase complex consists of several protein subunits and phospholipids in lamellar configuration [1]. Current efforts to elucidate the molecular organization of this membrane fragment proceed in two directions: (a) purification of membrane fragments with oligomycin-sensitive ATPase and (b) reconstitution of ATPase complex using purified components. The first approach yielded substantial progress recently [2, 3, 4]. In these and other studies, oligomycin-sensitivity has been the criterion distinguishing particulate from soluble ATPase. Whereas oligomycin-sensitivity is useful indicator of allotopic change in F_1-ATPase [5], it is well established not to reflect the native organization of ATPase complex. Thus, submitochondrial particles of different ATPase activity manifest the same sensitivity to oligomycin but a different F_1-ATPase response to aurovertin [6, 7]. This indicates variability in the

relationship between F_1-ATPase and the other components of complex. Although the influence of the ATPase inhibitor [8], of the oligomycin sensitivity-conferring protein [9, 10] and of phospholipids [11–14], on the catalytically active F_1-ATPase is known, a coherent picture on the overall organization of the native ATPase complex is still lacking.

An approach to this problem (cf. also ref. 15) is to produce gradual perturbations in membrane fragments in which the organization of the ATPase complex is as close as possible to that of intact mitochondria. MgATP submitochondrial particles [16] lend themselves to such studies since they have high phosphorylating efficiency and very low ATPase activity. The slow ATP hydrolysis is due either to the tight association of F_1-ATPase with its specific inhibitor or to the low proton permeability of membrane vesicles [17]. This study describes the effect of mild treatments on the properties of F_1-ATPase bound to MgATP particles.

MATERIAL AND METHODS

Diphosphatidylglycerol (cardiolipin), phospho*enol*pyruvate, ATP, MES (2 (*N*-morpholino)ethane sulfonic acid), hexokinase from yeast (Type C-300) of Sigma, and pyruvate kinase (solution in 50 % glycerol) of Boehringer have been used. Sonicated liposomes of diphosphatidylglycerol have been prepared as reported earlier [14].

MgATP submitochondrial particles were isolated from bovine heart mitochondria as described by Low and Vallin [16]. After the final washing, the particles were resuspended in 0.25 M sucrose, 10 mM Tris/MES (pH 7.4) at a concentration of 20 mg/ml and stored in aliquots at −40 °C. EDTA-particles [18] and ammonia-Sephadex particles (AS-particles, ref. 19) were prepared as described. The analytical procedures and the measurement of ATPase activity have been described previously [14]. Details are indicated in the table and figure legends.

Removal of ATPase inhibitor

Three procedures have been used: (a) EDTA-treatment: MgATP particles at a final concentration of 2 mg/ml were incubated in 250 mM sucrose, 10 mM Tris/MES, 10 mM EDTA (pH 7.4) 10 min at 37 °C. (b) Tris · HCl treatment: 2 mg/ml MgATP particles were incubated in 200 mM Tris · HCl, 150 mM sucrose (pH 7.4) 10 min at 37 °C. (c) Succinate, phosphate and P_i-acceptor-treatment (State-3 particles, ref. 17), 2 mg/ml MgATP particles were incubated in 10 mM KH_2PO_4, 10 mM succinate/Tris, 10 mM glucose, 20 units/ml hexokinase, 7 mM Tris/MES, 180 mM sucrose, 2 mM ADP, 4 mM $MgCl_2$ (pH 7.4) for 10 min at 37 °C. After incubation, the samples were centrifuged at 20 °C, 20 min at 40 000 rev./min and the sediments collected in 0.25 M sucrose, 10 mM Tris/MES (pH 7.4). State-3 particles were washed once.

Partial purification of ATPase inhibitor

The inhibitor protein released in the supernatant could be concentrated and purified as follows [20]: 50 % cold trichloroacetic acid was added drop-wise, yielding a final concentration of 10 %. The sample was centrifuged for 5 min at 18 000 rev./min and the sediment collected in 0.5 M Tris · HCl (pH 7.8) (0.1 ml/ml original supernatant). When necessary, the pH was adjusted further to neutrality and the sample centrifuged again to discard insoluble material. Additional purification has been

achieved through incubation of the supernatant for 90 s at 100 °C and removal of denaturated proteins by centrifugation. The final yield of inhibitor (treatment of MgATP particles with EDTA or Tris · HCl) was in the range of 1–3 μg/mg original MgATP particles.

Determination of residual ATPase inhibitor content

The ATPase inhibitor left in the particles was extracted and partially purified by sequential treatment with KOH and trichloroacetic acid [20]. The inhibitory effect has been tested on EDTA-particles.

RESULTS

In accordance with previous results [12, 14], high lability has been found in the organization controlling the ATPase activity of MgATP particles. In order to maintain a low ATPase activity, the concentration of chloride ions had to be minimal. As shown in ref. 14, 50 mM chloride anions added as Tris · HCl increased the ATPase activity by 60 %. Freezing and thawing did not change the ATPase activity but a 10-min preincubation at 37 °C in the absence of MgATP often sufficed to double the rate

TABLE I

RELEASE OF ATPase INHIBITOR FROM MgATP PARTICLES

MgATP particles were preincubated 10 min at 37 °C in 0.25 M sucrose, 10 mM Tris/MES (pH 7.4) with or without the additions indicated below, and centrifuged. For minor adjustments of preincubation medium with Tris · HCl and succinate, P_i plus P_i-acceptor, see Materials and Methods. Washing was omitted. Where indicated, the supernatants were collected and purified at the stage of trichloroacetic acid treatment (Materials and Methods). ATPase activity of sediments was measured in the absence (−) or in the presence (+) of a 5 μl aliquot of trichloroacetic acid-treated supernatant derived from the same particles. In order to test the effect of diphosphatidylglycerol, vesicles of this phospholipid were added to the incubation medium of ATPase assay at level of 0.5 mg diphosphatidylglycerol per mg of added particles. Conditions for ATPase assay 167 mM sucrose, 7 mM Tris/MES, 600 μg bovine serum albumin, 2.7 mM phospho*enol*pyruvate, 20 μg pyruvate kinase, 4 mM ATP, 4 mM $MgCl_2$ (pH 7.4). Final volume, 0.6 ml. After 10 min incubation at 37 °C, the reaction was stopped with 0.2 ml cold 50 % trichloroacetic acid.

Treatment of MgATP particles	Addition of supernatant	ATPase activity (μmol/mg per min)	
		Without diphosphatidylglycerol	With diphosphatidylglycerol
No preincubation	−	0.60	1.51
Preincubation			
Without additions	−	0.85	1.40
With 10 mM P_i	−	1.60	2.18
With 10 mM succinate	−	1.79	2.62
With 10 mM EDTA	−	2.10	2.77
	+	0.42	1.76
With 200 mM Tris · HCl	−	4.02	4.35
	+	0.45	2.27
With succinate, P_i	−	3.61	4.73
plus P_i acceptor	+	0.85	2.32

of ATP hydrolysis. Various agents, such as phosphate, succinate, EDTA, chloride anions (added as Tris · HCl), greatly enhanced the spontaneous increase of ATPase activity during preincubation (Table I). This finding is in agreement with a number of previous reports. These showed the activating effect of anions on submitochondrial particles [4, 21, 22], the inhibitory effect of anions on the association between F_1-ATPase and the inhibitor [8, 20] and the magnesium requirement for the association of ATPase inhibitor with the membrane [20]. Simultaneous addition of a P_i-acceptor system (yielding State-3 particles) increased the effectiveness of succinate and phosphate [17, 23]. In the presence of EDTA, Tris · HCl and succinate, P_i plus P_i-acceptor, the stimulation of ATPase activity coincided with a release of inhibitory material. The effect of this inhibitor was partially counteracted by diphosphatidylglycerol, an agent itself known to be antagonist of membrane-bound and isolated ATPase inhibitor [13, 14]. Consistently, the stimulation of ATPase activity, induced by diphosphatidylglycerol on MgATP particles (2.5-fold), was reduced after preincubation.

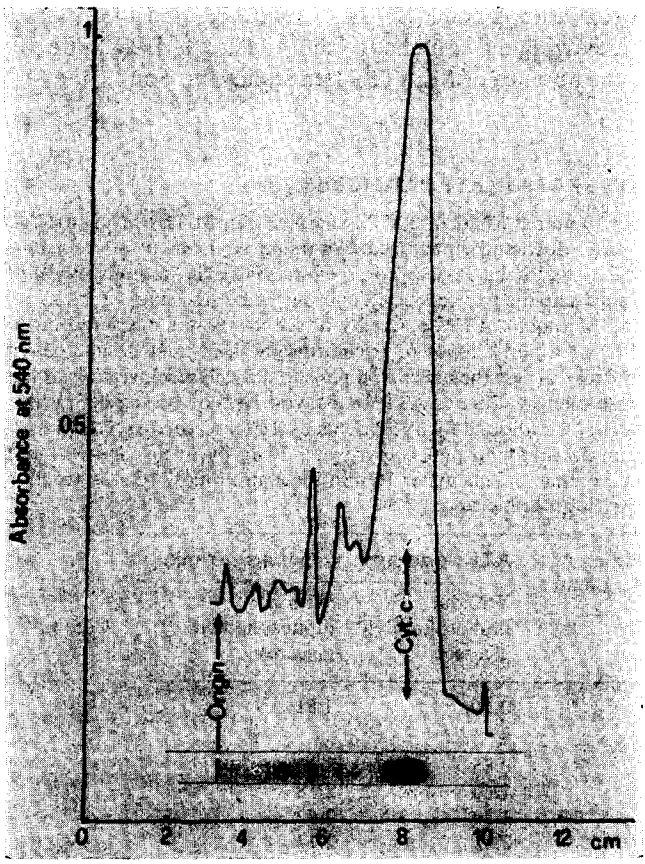

Fig. 1. Polyacrylamide gel electrophoresis of purified ATPase inhibitor. MgATP particles were incubated with 10 mM EDTA and the released ATPase inhibitor was purified at the stage of heat treatment as described under Materials and Methods. 30 μg of purified sample were subjected to electrophoresis [31] in 7.5 % acrylamide gels containing 0.1 % sodium dodecyl sulfate. The arrow indicates the position of a sample of cytochrome *c* run on a separate column.

In 10–15 different preparations of EDTA or Tris · HCl-treated MgATP particles and State-3 particles the effect of diphosphatidylglycerol ranged from no stimulation (cf. ref. 24) to a 50 % increase of ATPase activity. These higher figures of stimulation were more frequently observed on State-3 particles, indicating a residual control of ATPase activity by ATPase inhibitor in this preparation. FCCP stimulated these preparations (all derived from frozen-thawed MgATP particles) by 0–20 %. As shown before [17] MgATP particles retained low ATPase activity also in the conditions of high proton permeability. Parallel use of diphosphatidylglycerol and FCCP provided an useful criterion to assess the contribution of ATPase inhibitor and proton permeability in the control of ATPase activity. A clear manifestation of an oligomycin-sensitive diphosphatidylglycerol effect required the presence of serum albumin in the incubation medium.

Through trichloroacetic acid precipitation and heat treatment of the supernatant after preincubation of MgATP particles with EDTA or Tris · HCl, considerable purification of an inhibitor protein was achieved, the specific activity of which reached 2300 units/mg (see ref. 20 for definition of unit). Electrophoresis of the purified sample on polyacrylamide gel (Fig. 1) showed the presence of a major band that migrated in the same position as did cytochrome *c*. From these observations, it was concluded that the endogenous inhibitor [8] of the ATPase complex was liberated under these conditions.

The degree of inhibitor depletion could be suitable assessed by an estimate of the residual amount of ATPase inhibitor in the particles. Treatment with alkali released the residual ATPase inhibitor which was then partially purified by subsequent trichloroacetic acid precipitation. The results shown in Fig. 2 show that a surprisingly

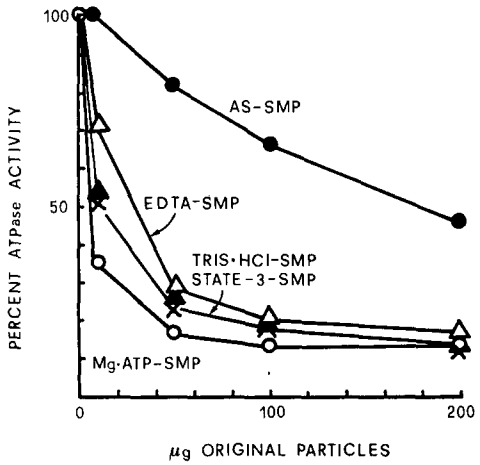

Fig. 2. Residual ATPase inhibitor in depleted particles. 0.1 ml 1 M KOH was added to 5 mg particles suspended in 1.0 ml cold water. After 1 min at 0 °C, 0.2 ml 50 % trichloroacetic acid were added and the sample centrifuged 5 min at 13 000 rev./min. Sediments were resuspended in 0.5 ml 0.5 M Tris · HCl, pH 7.8, and centrifuged again. Supernatants collected and tested for inhibitory effect on ATPase activity of EDTA-particles (20 μg) in the conditions described in Table I. The ATPase activity of original particles (μmol ATP splitting/mg per min) was: MgATP particles, 0.50; AS-particles, 8.4; EDTA-particles, 3.6; State-3 particles, 4.1; Tris · HCl-treated MgATP particles, 4.6. The data on the abscissa refer to the amount of particles represented by the aliquot of final extract added to the incubation medium.

high amount of ATPase inhibitor was present in apparently depleted particles. 50 % of the maximal inhibition was obtained with an amount of final extract corresponding to 7 μg original MgATP particles. This amount increased to only 10 μg particles when the ATPase was stimulated 7–9 fold by pretreatment with Tris · HCl or succinate, phosphate and P_i-acceptor. A similar determination on EDTA-particles showed that 50 % of maximal inhibition was produced by 17 μg particles indicating a significant content of ATPase inhibitor also in this case. By contrast AS-particles were effectively depleted.

Isolated F_1-ATPase showed a greater resistance to cold inactivation when associated with ATPase inhibitor [8]. Experiments were done to see whether membrane-bound ATPase retained cold stability when partially or completely depleted of ATPase inhibitor. According to the data in Table II, at 0 °C, the stability of MgATP

TABLE II

COLD LABILITY OF DEPLETED PARTICLES

MgATP particles were treated with EDTA, Tris · HCl or succinate, P_i and P_i-acceptor as described under Materials and Methods. The ATPase activity was assayed as described in Table I with 15–30 μg particles (50–100 μg MgATP particles) and the additions indicated below. Preincubation: (a), the tubes containing the components of incubation medium minus ATP and Mg were stored 60 min at 0 °C before starting the reaction at 37 °C with 4 mM MgATP. (b) The tubes containing all components of incubation medium were stored 60 min at 0 °C and then transferred at 37 °C. During this preincubation, very limited ATP splitting was recorded. At 0 °C, the pH of the medium was 7.6. Where indicated, glycerol (20 %, w/v) and NaCl (0.1 M) were added before starting the preincubation at 0 °C.

Particles	ATPase activity (μmol/mg per min)		
	Without preincubation	Preincubation without MgATP	Preincubation with MgATP
MgATP particles	0.63	0.59	0.47
EDTA-treated MgATP particles	2.18	2.10	1.33
Tris · HCl-treated Mg-ATP particles	3.87	3.79	2.27
State-3 particles	3.47	3.63	2.94
EDTA-particles	4.90	4.39	1.87
AS-particles	6.88	6.02	3.33
AS-particles + glycerol	5.80	6.12	6.02
AS-particles + NaCl	7.41	5.37	2.15

particles treated with EDTA or Tris · HCl was significantly impaired when incubation in the cold was done in the presence of MgATP. The same effect of low temperature in the presence of MgATP was seen on EDTA or AS-particles. Time course experiments showed that the decrease of ATPase activity at 0 °C was slow and progressive, although more rapid in the first 30 min. Loss of activity was 40–50 % after 1 h storage at 0 °C and 60 % after 2 h. Dilution of the particles and omission of albumin from the incubation medium facilitated to some degree the inactivation. Oligomycin sensitivity was always maintained, indicating that solubilization of F_1-ATPase with subsequent cold inactivation did not take place. MgATP particles and especially State-3 particles were inactivated to a lesser degree (from 15 to 37 % after 1 h storage in 6 determina-

tions). Only on these two preparations did diphosphatidylglycerol produce a significant stimulation of ATPase activity after preincubation at 0 °C, demonstrating that the decrease of ATPase activity was in these cases partly due to reassociation of F_1-ATPase with residual ATPase inhibitor during prolonged storage in the cold. Short-time preincubation with MgATP at 25 °C did not produce reassociation of ATPase inhibitor in State-3 particles [17]. Addition of glycerol before exposure to cold prevented the inactivation which was instead increased by salts. Some salt-induced inactivation was also seen in the absence of MgATP. The respective effects of glycerol and salts on the cold inactivation were similar in particles and in isolated F_1-ATPase [25–27], suggesting a similarity between the two phenomena. Protection by glycerol against cold inactivation of isolated enzyme from chloroplasts was in part attributed to prevention of aspecific aggregation [26]. Inspection of several samples with variable amounts of particles showed that no aggregation occurred after preincubation at 0 °C with MgATP.

Fig. 3 demonstrates the great effectiveness of MgATP in promoting cold inactivation. The effect was already very significant at 0.01 mM and almost maximal at 0.05 mM. The further increase seen at much higher MgATP concentrations is attributable to salt effect. These values can be compared with the MgATP-induced conformational change of submitochondrial particles detectable at 0.2 mM concentration [28]. Magnesium alone was ineffective, whereas ATP alone promoted some inactivation. When ADP, GTP, UTP and ITP replaced ATP, a slight or no effect by these nucleotides was found.

Table III shows that cold-induced inactivation was stable since reisolated particles showed decreased ATPase activity. During these experiments, it was observed that some inactivation was produced by MgATP also after preincubation at room temperature. This effect was less manifest than that produced in the cold (with AS-particles the inhibition by 4 mM MgATP was 35 % at 20 °C and 65 % at 0 °C). Rapid isolation of F_1-ATPase from cold inactivated AS-particles by treatment with diphos-

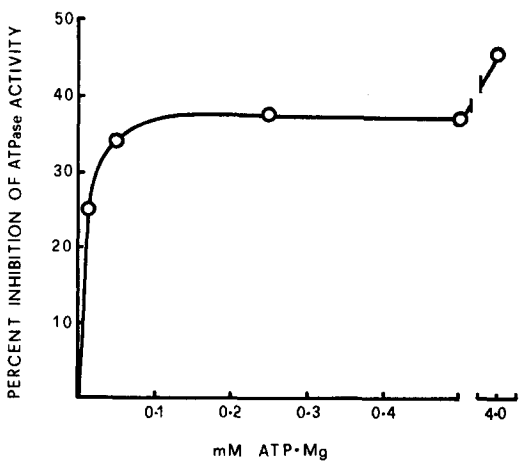

Fig. 3. Effect of MgATP concentration. 15 μg AS-particles were preincubated 60 min at 0 °C with the indicated amount of MgATP in the conditions described in Table II. After preincubation, enough MgATP was added to bring the final concentration to 4 mM.

TABLE III

F_1-ATPase FROM COLD-INACTIVATED PARTICLES

0.6 mg AS-particles were preincubated 60 min at O °C in 6.0 ml of the medium used for ATPase assay as described in Table I, with or without the addition of MgATP. Where indicated, glycerol was added at 20 % (w/v) concentration and sucrose was reduced to 125 mM. After preincubation the particles were sedimented 20 min at 40 000 rev./min and resuspended in 0.3 ml of 0.25 M sucrose, 10 mM Tris/MES (pH 7.4). F_1-ATPase was extracted by 5 min incubation at 37 °C with diphosphatidylglycerol 0.5 mg/mg protein [14]. After incubation with diphosphatidylglycerol, the samples were centrifuged 30 min at 50 000 rev./min and the oligomycin-insensitive ATPase activity measured in the supernatant.

Preincubation of particles	ATPase activity (μmol/mg per min)	
	Sedimented particles	Solubilized F_1-ATPase
60 min at 0 °C without MgATP	9.3	21.6
60 min at 0 °C with 4 mM MgATP	4.2	14.9
60 min at 0 °C with MgATP plus glycerol	7.9	24.8

phatidylglycerol [14, 29] yielded a soluble preparation with lower activity, supporting the possibility of inactivation of F_1-ATPase during cold exposure.

DISCUSSION

Attempts to remove the ATPase inhibitor from MgATP particles results in the release of some inhibitor in the surrounding medium, and its dislocation in other sites of membrane. Preliminary reports [24] have shown that the F_1-ATPase isolated from inhibitor-deficient particles was highly active, although significant amounts of ATPase inhibitor were retained. This suggested that a part of dislocated ATPase inhibitor was still bound to the F_1-ATPase.

Similarly, inhibitor dislocation at non-operative sites were indicated in MgATP particles [17]. It is justified to conclude that in order to control aberrant ATPase activity, the inhibitor protein has to be included in a proper combination with other components of ATPase complex, thus ensuring the correct association of F_1-ATPase with the membrane and the appropriate interaction with external ligands. Dislocation of ATPase inhibitor from its physiological sites appears to be very easy in MgATP particles.

EDTA strongly enhances the disassembly of the ATPase complex, suggesting that magnesium has a great part in sustaining the native organization. Experiments in progress indicate a strong decrease of magnesium content in particles which are not under the control of residual ATPase inhibitor. This is in line with a previous report on the release of magnesium from mitochondrial inner membrane [30].

The appearance of high ATPase activity allows the possibility of detecting cold-lability in membrane-bound F_1-ATPase. Cold inactivation of membrane-bound F_1-ATPase requires the presence of ATP, but is greatly enhanced by simultaneous addition of magnesium. The process is slow, accelerated by salts and prevented by glycerol. These distinctive features indicate some interesting similarities with the cold-lability of soluble enzyme.

The MgATP complex is known to induce significant conformational changes in the mitochondrial inner membrane [28] and in membrane-bound F_1-ATPase [6]. It is conceivable that addition of ATPMg to particles under conditions that do not permit rapid ATP hydrolysis (0 °C) might destabilize membrane-bound F_1-ATPase, producing a form of enzyme apt to be inactivated by cold. Interestingly, Rosing et al. [27] have postulated the occurrence of a reversible conformational transition of membrane-bound F_1-ATPase during oxidative phosphorylation leading to the 9-S form of enzyme. This form can be converted at low temperature into irreversibly denaturated 3–S form when the enzyme is in solution. Conversion into 3-S form might occur in the membrane when F_1-ATPase is no longer protected by a tight organization of ATPase complex and when the splitting of MgATP at sufficient rapid rate is prevented. When this work was completed, a communication describing the effect of preincubation with MgATP on ammonia-EDTA particles appeared [32]. The results are in agreement with our findings on AS (ammonia-Sephadex) particles.

ACKNOWLEDGMENTS

This work received financial support from Consiglio Nazionale delle Ricerche (C.N.R.) Contr. No 75.00497.04/115.3488.

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