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F_1-ATPase FROM DIFFERENT SUBMITOCHONDRIAL PARTICLES

A. BRUNI, A. PITOTTI, P. PALATINI, F. DABBENI-SALA and E. BIGON

Institute of Pharmacology, University of Padova, Largo E. Meneghetti 2, and C.N.R. Unit for the Study of Physiology of Mitochondria, Via Loredan 16, 35100 Padova (Italy)

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Summary

1. F_1-ATPase has been extracted by the diphosphatidylglycerol procedure from mitochondrial ATPase complexes that differ in ATPase activity, cold stability, ATPase inhibitor and magnesium content.

2. The ATPase activity of the isolated enzymes was dependent upon the activity of the original particles. In this respect, F_1-ATPase extracted from submitochondrial particles prepared in ammonia (pH 9.2) and filtered through Sephadex G-50 was comparable to the enzyme purified by conventional procedures (Horstman, L.L. and Racker, E. (1970) J. Biol. Chem. 245, 1336—1344), whereas F_1-ATPase extracted from submitochondrial particles prepared in the presence of magnesium and ATP at neutral pH was similar to factor A (Andreoli, T.E., Lam, K.W. and Sanadi, D.R. (1965) J. Biol. Chem. 240, 2644—2653).

3. No systematic relationship has been found in these F_1-ATPase preparations between their ATPase inhibitor content and ATPase activity. Rather, a relationship has been observed between this activity and the efficiency of the ATPase inhibitor-F_1-ATPase association within the membrane.

4. It is concluded that the ATPase activity of isolated F_1-ATPase reflects the properties of original ATPase complex provided a rapid and not denaturing procedure of isolation is employed.

Abbreviations: AS particles, submitochondrial particles prepared in ammonia solution (pH 9.2) and subsequently filtered through Sephadex G-50; Mg-ATP particles, submitochondrial particles prepared in the presence of magnesium and ATP at neutral pH; EDTA particles, submitochondrial particles prepared in the presence of EDTA at pH 8.5; MES, 2-(*N*-morpholino)ethanesulfonic acid.

Introduction

Native ATPase complex included in the mitochondrial membranes has low ATPase activity. This property is required to preserve newly formed ATP when energy can be derived from oxidation of appropriate substrates [1—3]. In contrast with chloroplasts [4] and several bacteria [5—8], the F_1 portion of mitochondrial ATPase complex is usually obtained in a form of high ATPase activity. However, the possibility that this results from the isolation and purification of the enzyme has been considered since its discovery [9]. In line with this possibility a soluble preparation of mitochondrial F_1-ATPase with latent ATPase activity has been obtained [10]. The latent hydrolyzing activity of ATPase complex is believed to result from an ATP · Mg dependent reversible association between the F_1-ATPase and natural ATPase inhibitors that were first identified in bovine-heart mitochondria [1] and subsequently in chloroplasts [11], yeast mitochondria [12,13] and bacteria [5,7]. In sonicated submitochondrial particles, coupled respiration loosens the association [14] but the reassociation becomes more difficult even if the endogenous inhibitor is retained in the membranes [15,16]. Depending on the procedure of preparation, the damage to this mechanism of regulation in sonicated particles can progress untill a complete loss of the ATPase inhibitor [3]. This is not without consequence for the ATPase complex since in resolved particles, membrane-bound F_1-ATPase becomes cold labile in the presence of ATP · Mg [16]. Previously [17,18] it has been found that after a short time of incubation with diphosphatidylglycerol, highly purified F_1-ATPase is liberated from submitochondrial particles and recovered in the supernatant whereas all phospholipid vesicles are bound to the insoluble membrane. The same method is used here in order to investigate whether the alteration of the ATPase complex composition is reflected in the properties of isolated F_1-ATPase.

Materials and Methods

Bovine-heart diphosphatidylglycerol (cardiolipin) obtained from Sigma in ethanol solution yielded only a single spot in thin-layer chromatography. The phospholipid solution was taken to dryness and sonicated after addition of 0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA (pH 7.4) to yield a transparent dispersion of 5—6 mg/ml.

Bovine heart Mg-ATP particles [19], EDTA particles [20], and AS particles [21] were prepared as originally described. After the final washing in 0.25 M sucrose, 10 mM $MgCl_2$, Mg-ATP particles were resuspended in 0.25 M sucrose, 10 mM Tris-MES (pH 7.4). The incubation of these particles with 10 mM EDTA or 200 mM Tris-HCl or succinate, P_i plus P_i-acceptor (State 3 particles, ref. 14, 15) has been described previously [16].

Isolation of F_1-ATPase. Submitochondrial particles (0.5—8 mg) have been incubated for 5 min at 37°C with 0.5 mg diphosphatidylglycerol/mg protein in 4 mM ATP, 0.25 M sucrose, 10 mM Tris-MES (pH 7.4). The final concentration of particles was 1.25 mg/ml. The incubation was followed by 30 min centrifugation at 20°C at 50 000 rev./min (150 000 × *g*), and the supernatant centrifuged again to remove any residual membrane fragments. When desired, the

clear supernatant was concentrated to a small volume with either immersible molecular separators (Millipore Co., retention for molecules above 10 000 dalton), or with Amicon membrane cones, type GF 50 A (retention for molecules above 50 000 dalton). Fractionation with ammonium sulfate followed a procedure described earlier [18], but any exposure to cold temperature was avoided. Instead of EDTA and Tris-HCl, 10 mM Tris-MES (pH 7.4) was employed throughout all procedures.

Residual ATPase inhibitor in purified F_1-ATPase [3]. 0.05 ml 1 M KOH was added to 0.5 ml cold water containing 30—60 μg F_1-ATPase isolated from the indicated particles and fractionated with ammonium sulfate. After 1 min at 0°C, 0.1 ml 50% trichloroacetic acid was added and the sample centrifuged for 5 min at 13 000 rev./min. The sediment was resuspended in 0.1 ml 0.5 M Tris-HCl (pH 7.8) and centrifuged again. Aliquots of the supernatant were collected and added to the incubation medium used for the determination of the ATPase activity. 20 μg EDTA particles were used in this assay.

The measurement of ATPase activity is described in the legend to Table I. Proteins were determined with bovine serum albumin as standard [22]. Deoxycholate served to dissolve particulate material. Dilute protein solution was concentrated with 10% trichloroacetic acid before determination [23]; this procedure was also useful to eliminate interferring compounds. Polyacrylamide gel electrophoresis in the absence [24] or in the presence of 0.1 M sodium dodecyl sulfate [25] was done as described earlier. The sample gel [24] was omitted. Gels were stained with 0.25% Coomassie blue in 50% methanol/ 10% glacial acetic acid (v/v) for 5 h. Destaining was obtained by spontaneous leakage of the dye in 7.5% acetic acid/5% methanol.

Magnesium was extracted 120 min at 0°C with 0.5 M perchloric acid from particles which were previously sedimented and washed once in 0.25 M sucrose, 10 mM Tris-MES (pH 7.4) after the complete procedure of preparation. Two additional washings were performed as indicated. Magnesium determination at 284 nm was done in the presence of 36 mM LaO_3 with a Perkin-Elmer model 305 Atomic Absorption Spectrophotometer. ATP and ADP were extracted for 15 min at 0°C with 0.33 M perchloric acid/1.5 mM EDTA, from particles washed once. After centrifugation the perchloric extracts were neutralized with 0.1 M Tris in 10% KOH. ADP and ATP were quantified fluorimetrically by enzymatic procedures [26].

Results

ATPase activity of particulate and soluble preparations

Table I shows that the ATPase activity of the submitochondrial particles used in this study was widely different and ranged from a minimal value in Mg-ATP particles to a maximal value in AS particles. Since the diphosphatidylglycerol procedure was used to solubilize F_1-ATPase from these particles it was necessary to test whether the phospholipid would maintain the different activity among the ATPase complexes. Diphosphatidylglycerol was reported [16,18, 27,28] to modify the ATPase activity of submitochondrial particles by releasing the association between F_1-ATPase and the endogenous ATPase inhibitor [1]. To prevent solubilization of F_1-ATPase during this assay serum albumin

TABLE I

ATPase ACTIVITY OF SUBMITOCHONDRIAL PARTICLES

Each tube contained in a final volume of 0.6 ml 167 mM sucrose, 7 mM Tris-MES, 600 μg bovine serum albumin, 2.7 mM phospho*enol*pyruvate (monopotassium salt), 20 μg pyruvate kinase, 4 mM ATP, 4 mM $MgCl_2$, Mg-ATP particles, 100 μg (other particles, 15—25 μg) pH 7.2 at 37°C. Incubation 10 min at 37°C. The reaction was stopped by the addition of 0.2 ml cold 50% trichloroacetic acid. The values are means ±S.E. of 6—20 determinations. When required the diphospatidylglycerol sonicated dispersion was added to the incubation mixture to reach a concentration of 0.5 mg/mg particle. Where indicated Mg-ATP particles were preincubated for 10 min at 37°C with 10 mM EDTA or 200 mM Tris-HCl, centrifuged and resuspended in 0.25 M sucrose, 10 mM Tris-MES (pH 7.4) (ref. 16).

Preparation	Addition of diphosphatidyl-glycerol	ATPase activity (μmol/mg per min)	Relative ratio of ATPase activity (Mg-ATP particles = 1)
Mg-ATP particles	—	0.6 ± 0.02	1
	+	1.5 ± 0.04	1
Mg-ATP, preincubated with EDTA	—	2.0 ± 0.11	3.3
	+	2.8 ± 0.11	1.9
Mg-ATP, preincubated with Tris-HCl	—	3.8 ± 0.18	6.3
	+	4.6 ± 0.35	3.1
State 3 particles	—	3.8 ± 0.31	6.3
	+	4.9 ± 0.31	3.3
EDTA particles	—	5.0 ± 0.12	8.3
	+	5.3 ± 0.16	3.5
AS particles	—	9.2 ± 0.55	15.3
	+	8.6 ± 0.60	5.7

was added to the incubation medium. Under these conditions high oligomycin sensitivity (approx. 80%) was maintained. Except for the oligomycin sensitivity, the effect of diphosphatidylglycerol on the ATPase activity was not affected by serum albumin. In agreement with previous studies [16,18] the phospholipid yielded a sharp increase of ATPase activity in Mg-ATP particles. However, this effect was lower or absent in other particles reflecting the decreased influence of the ATPase inhibitor in these ATPase complexes. In spite of diphosphatidylglycerol-induced stimulation the values of the ATPase activity among the particles remained significantly different. In a previous study [16] it was found that the high ATPase activity of the preparations reported in Table I did not always correlate with a low content of the endogenous ATPase inhibitor. Rather, it was difficult to achieve reassociation of F_1-ATPase with the ATPase inhibitor left in the membrane. Since Mg^{2+} and ATP are needed for this association [3], possible differences in the Mg and adenine nucleotide content were investigated. A variation in the composition of ATPase complexes would have been of value also for the assessment of different properties in the respective F_1-ATPase. As shown in Table II, the amount of Mg^{2+} was high only in Mg-ATP and State 3 particles prepared in the presence of this cation. The value of 30—36 nmol/mg protein was similar to the one reported for electron transport particles prepared in the presence of Mg^{2+} [29]. Additional washings lowered the magnesium content of Mg-ATP and State 3 particles to a constant level of about 50% of the initial amount without any substantial change in ATPase activity. In spite of this decrease the cation

TABLE II

MAGNESIUM AND ADENINE NUCLEOTIDE CONTENT OF SUBMITOCHONDRIAL PARTICLES

Magnesium content: Particles were washed once and extracted with 0.5 M perchloric acid 2 h at 0°C. After centrifugation the magnesium content was determined in the supernatants. Means ± S.E. of 3—11 determinations. ATP, ADP content: Washed particles were extracted with 0.33 M perchloric acid and 1.5 mM EDTA 15 min at 0°C. After centrifugation the supernatants were tested for ATP and ADP content by fluorimetric procedures. Means ± S.E. of 2—5 determinations. When indicated Mg-ATP particles were preincubated 10 min at 37°C with 10 mM EDTA or 200 mM Trsi-HCl as outlined in Table I and ref. 16.

Particles	Mg^{2+} content (nmol/mg)	ATP content (nmol/mg)	ADP content (nmol/mg)
AS particles	3.5 ± 1.4	—	—
EDTA particles	5.4 ± 0.9	0.75 ± 0.07	0.73 ± 0.04
State 3 particles *	30.4 ± 1.8	1.86 ± 0.13	0.90 ± 0.07
Mg-ATP particles *	35.9 ± 2.3	0.91 ± 0.08	0.90 ± 0.07
Mg-ATP preincubated with EDTA	8.8 ± 2.4	1.14 ± 0.01	1.19 ± 0.24
Mg-ATP preincubated with Tris-HCl	10.0 ± 1.7	0.97 ± 0.06	0.90 ± 0.01

* After two additional washings the Mg^{2+} content of Mg-ATP particles decreased to 16.2 ± 2.1, that of State 3 particles to 15.3 ± 2.7. The ATP content of State 3 particles decreased to 0.96 nmol/mg.

content remained 2—3 times higher than that of the other particles. Incubation of Mg-ATP particles with EDTA produced a significant increase in ATPase activity and decreased the Mg^{2+} content to a low level. Incubation with Tris-HCl had similar effects. All other particles were highly magnesium-deficient.

Adenine nucleotide content determinations did not show significant differences among the particles. State 3 particles have a higher ATP content, but this difference disappeared upon repeated washing, indicating that the ATP was loosely bound or was present in the internal compartment. The final amounts of adenine nucleotides detected were in agreement with those recently reported [30].

In accordance with earlier results [18], Table III shows that the differences in the activity of submitochondrial particles were reflected in the ATPase activities of isolated F_1-ATPases. As expected, the relative ratio among the activity of the preparations of F_1-ATPase corresponded within experimental variation to that of the particles tested in the presence of diphosphatidylglycerol. This showed that activation of the enzyme by the phospholipid was produced during the isolation and was greater in the case of Mg-ATP particles. The differences among the F_1-ATPase remained significant after fractionation with ammonium sulfate or after concentration of dilute enzymes with Amicon membrane cones retaining proteins of molecular weight above 50 000 and, therefore, expected to remove unbound ATPase inhibitor. No differences in cold lability were observed among the soluble preparations. The increase in activity following ammonium sulfate fractionation was, in part, due to further purification of the enzyme. Direct stimulation of activity in freshly isolated F_1-ATPase was observed after the addition of 1 mM $(NH_4)_2SO_4$ or KCl to the incubation medium used for the ATPase assay. This was in keeping with a number of recent reports, showing the activating effect of several anions on soluble and membrane-bound F_1-ATPase from various sources [31—36]. Activating anions are expected to induce a change in the enzyme configuration

TABLE III

F_1-ATPase FROM DIFFERENT PARTICLES

1—5 mg particles were incubated for 5 min at 37°C with 0.5 mg/mg diphosphatidylglycerol in 0.25 M sucrose, 10 mM Tris-MES, 4 mM ATP (pH 7.4). The particle concentration was 1.25 mg/ml. The samples were centrifuged twice for 30 min at 20°C at 50 000 rev./min. Where indicated the final supernatants were concentrated with Amicon membrane cones (CF 50 A, 95% retention for molecules above 50 000 dalton) or fractionated with saturated ammonium sulfate. The ATPase activity was determined as described in Table I with 1—5 μg enzyme. The values are means ± S.E. of about 10 determinations. The ATPase activity of original particles was as reported in Table I. Preincubation of Mg-ATP particles with Tris-HCl, Table I and ref. 16.

Original particles	Activity of isolated F_1-ATPase (μmol ATP hydrolyzed/mg per min)		
	After isolation	After concentration	After salt fractionation
AS particles	41.0 ± 4.5	57.8 ± 1.8	84.4 ± 9.7
EDTA particles	20.2 ± 3.0	28.5 ± 2.2	39.6 ± 3.5
State 3 particles	18.0 ± 1.9	24.1 ± 0.4	38.0 ± 5.4
Mg-ATP particles	6.0 ± 0.3	8.0 ± 0.5	17.1 ± 1.3
Mg-ATP particles *	16.0 ± 1.8	23.8 ± 1.5	41.6 ± 3.1
Mg-ATP particles preincubated with 200 mM Tris-HCl	11.7 ± 2.2	27.9 ± 0.9	41.9 ± 3.3

* 5 mM EDTA present at moment of F_1-ATPase detachment.

by binding to regulatory sites [33,35]. Table III also shows that when EDTA was added to Mg-ATP particles during F_1-ATPase detachment, the isolated enzyme exhibited higher activity. Separate experiments demonstrated that this effect required EDTA during detachment of the enzyme. Incubation of low activity F_1-ATPase with EDTA did not increase ATPase activity. An activating effect of EDTA was also reported during the extraction of F_1-ATPase by the chloroform procedure [37]. The activation by EDTA during the isolation of F_1-ATPase from Mg-ATP particles, prompted experiments to establish whether or not any denaturation of the enzyme occurred in the absence of this chelating agent. The inactivation of F_1-ATPase in this particular case could originate from the high Mg^{2+} content of the membrane. However, incubation of F_1-ATPase preparations in the presence of high Mg^{2+} concentration (4 mM) failed to produce irreversible ATPase inhibition. Similarly, repeated washing of Mg-ATP particles before isolation of the enzyme did not increase the activity of resulting F_1-ATPase, in spite of the decrease in the Mg^{2+} content (Table I). Furthermore and in agreement with previous observations [18], unmasking of the ATPase activity followed storage at room temperature of purified F_1-ATPase from Mg-ATP particles in the presence of residual ammonium sulfate. At the end of a 20-h period, the activity of this preparation rose by 150% and was almost indistinguishable from that of the other F_1-ATPases which were activated much less under the same conditions (25—50% depending upon the original activity). From these experiments it was concluded that F_1-ATPase from Mg-ATP particles was not denatured in the absence of EDTA but retained its latent ATPase activity.

Purity and ATPase inhibitor content of isolated F_1-ATPase

A recent paper [38] reported the presence of NADH dehydrogenase contamination in F_1-ATPase after incubation of particles with diphosphatidylglycerol. In our work appropriate assays demonstrated that negligible NADH-ferricyanide reductase activity was present in all preparations. This was likely to be due to the brief exposure of particles to the phospholipid dispersion under the conditions employed. Some differences among the various F_1-ATPase preparations emerged when their purity was tested by polyacrylamide gel electrophoresis in the absence of denaturing agents. As shown in Fig. 1A, the supernatant from Mg-ATP particles was resolved in three major and two diffuse bands. The first (R_F, 0.29) corresponded to the ATPase; the second (R_F 0.39) was sometimes split into two components and a trace of it appeared also when the release of F_1ATPase was prevented by the omission of diphosphatidyl-

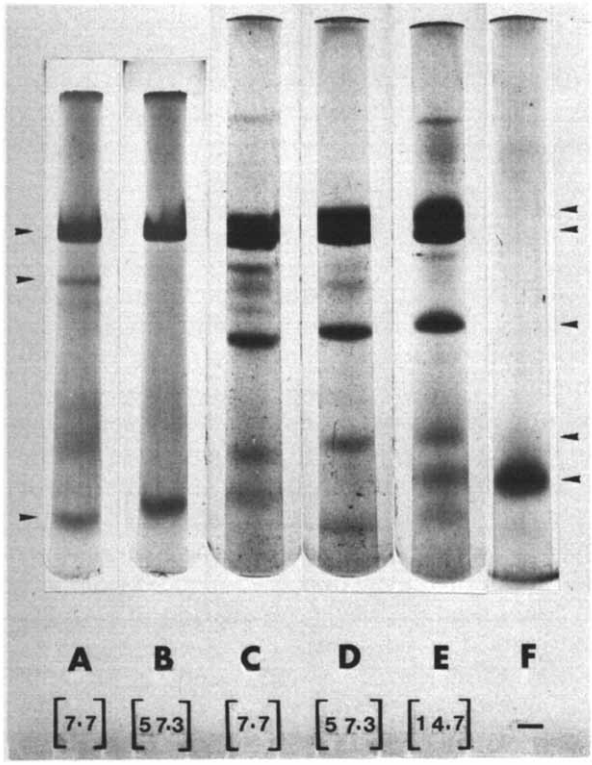

Fig. 1. Polyacrylamide gel of isolated F_1-ATPases. The supernatants after incubation of particles with diphosphatidylglycerol were either concentrated by membrane cones or further purified by ammonium sulfate fractionation. Electrophoresis under not dissociating conditions (gels A, B) was performed [24] in 7% acrylamide, omitting the sample gel. Electrophoresis under dissociating conditions (gels, C, D, E, F) was performed [25] in 10% acrylamide, 0.1% sodium dodecyl sulfate; the samples were prepared in the presence of 0.5% sodium dodecyl sulfate and 5% (v/v) β-mercaptoethanol. Gels A—E were loaded with 30 μg protein. A, F_1-ATPase from Mg-ATP particles; B, F_1-ATPase from AS particles, both after simple isolation and concentration; C and D, the same preparations under dissociating conditions; E, F_1-ATPase from Mg-ATP particles purified by ammonium sulfate fractionation. Gel F contained 10 μg ATPase inhibitor [1] purified as described [46]. The numbers in parenthesis refer to the specific ATPase activity of preparations (μmol/mg per min).

glycerol from the incubation mixture. The third (R_F 0.88) was seen occasionally, and it probably corresponded to subunits of F_1-ATPase dissociated in the absence of stabilizing agents [39,40]. Salt fractionation removed the contaminations and yielded profiles equal to those in the supernatant from AS particles (Fig. 1B). From these particles the enzyme was extracted in highly purified form since only the first band, and occasionally the third, were present. Considering the limited degree of contamination and the persistence of different ATPase activity after $(NH_4)SO_4$ fractionation it was concluded that purification of the enzyme was not responsible for its variable activity.

When the supernatant from Mg-ATP particles was subjected to electrophoresis under dissociating conditions (Fig. 1C) three to four bands were present in addition to the five subunits of F_1-ATPase. These were located between the β- and γ- subunits and therefore had a molecular weight between 33 000 and 51 000 [41]. The same pattern was shown by the preparation from EDTA particles. These bands were either absent or reduced in number and staining intensity in the supernatants from AS particles, State 3 particles and Mg-ATP particles preincubated with Tris-HCl (Fig. 1D).

Additional peptides of approx. molecular weight 40 000 with inhibitory effect on F_1-ATPase were described in a preparation of enzyme from yeast [42]. Peptides of similar molecular weight were observed in F_1-ATPase from brown adipose tissue [43] and in ATPase complexes from various sources

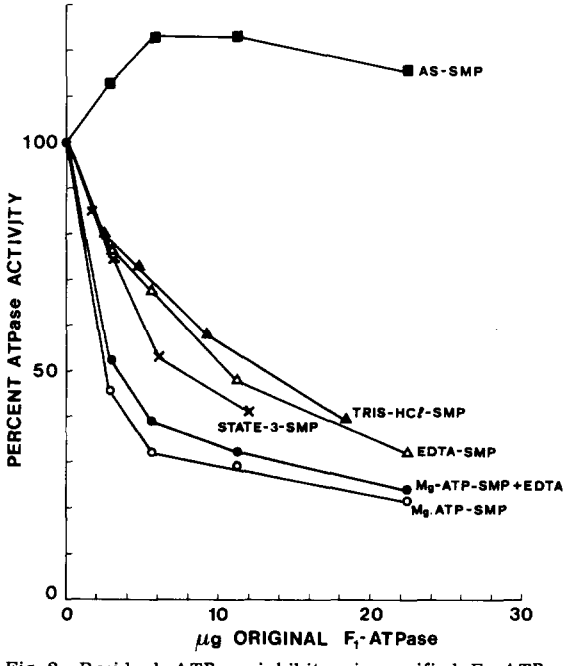

Fig. 2. Residual ATPase inhibitor in purified F_1-ATPases. Experimental procedures as described in Material and Methods. The data on the abscissa refer to the amount of F_1-ATPase represented by the aliquot of final extract added to the incubation medium. The particles from which F_1-ATPase was isolated are indicated. Mg-ATP-SMP + EDTA denotes the F_1-ATPase isolated from Mg-ATP particles in the presence of 5 mM EDTA. Tris-HCl-SMP denotes the F_1-ATPase extracted from Mg-ATP particles previously incubated with 200 mM Tris-HCl [16].

(reviewed in ref. 44). On this basis it cannot be excluded that additional components influencing the activity of F_1-ATPase in Mg-ATP and EDTA particles are extracted by diphosphatidylglycerol along with the enzyme. Salt fractionation almost entirely removed the extra peptides (Fig. 1E). After this purification procedure a major difference among the preparations was the presence in F_1-ATPase from Mg-ATP particles of the subunit corresponding to the ATPase inhibitor (Fig. 1F). The appearance of the band at the end of the run was not constant and did not allow conclusions pertaining to the proposed difference between the ϵ-subunit of F_1-ATPase and the ATPase inhibitor [45].

For the extraction of ATPase inhibitor from the F_1-ATPase preparations the most suitable procedure was the sequential treatment with KOH and trichloroacetic acid [3]. This procedure was applied to F_1-ATPase preparations purified at the stage of ammonium sulfate fractionation in order to avoid the possible interference by the ATPase inhibitor which might be released during incubation with phosphatidylglycerol. Fig. 3 shows high ATPase inhibitor content in the F_1-ATPase isolated from Mg-ATP particles either in the presence or in the absence of EDTA. This was particularly striking in view of the different activity of the two preparations. An appreciable but reduced ATPase inhibitor content was found in F_1-ATPase from State 3, EDTA and Mg-ATP particles previously incubated with Tris-HCl. In the preparation from AS particles no inhibitor was detected and a definite stimulation of ATPase activity followed the addition of the extract from denatured enzyme. The ATPase inhibitor content of particles [16] and of isolated F_1-ATPase showed a significant correlation.

Discussion

A rapid and not denaturing procedure allows a useful comparison among F_1-ATPases originating from different submitochondrial particles. A comparison is also possible between the properties of the enzyme before and after isolation.

Because of phosphorylating capacity and low ATPase activity [19], Mg-ATP particles were considered to retain the native organization of the ATPase complex. F_1-ATPase extracted from these particles has low ATPase activity. Progressive resolution of the ATPase complex components leads to increased ATPase activity and hence to more active isolated enzyme. Earlier studies [1—3,14] established the role of the ATPase inhibitor in the regulation of the catalytic activity of F_1-ATPase. As shown by gel electrophoresis and biological assay the content of this peptide in the various preparations cannot entirely explain their different activity. Furthermore, EDTA, an agent that can release the association between the F_1-ATPase and the ATPase inhibitor [16], increases the activity of the isolated enzyme when added before detachment from the membrane, but not after isolation. This indicates that it is the tightness of the association between F_1-ATPase and the ATPase inhibitor within the membrane which determines whether the isolated enzyme retains high or low ATPase activity. F_1-ATPase obtained from membranes in which the association is not efficient can have high activity also in spite of a large amount of inhibitor present. This is in accord with the comparable ATPase inhibitor content in active F_1-ATPase and in factor A of low activity [47] and with the

existence of non-inhibited F_1-ATPase-inhibitor complex [14,11]. The EDTA effect confirms the role of membrane-bound Mg^{2+} in the maintenance of the ATPase complex organization [16] and is in keeping with the high level of this cation found in Mg-ATP particles.

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