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STABILIZATION OF RAT LIVER MITOCHONDRIAL F_1-ADENOSINE TRIPHOSPHATASE DURING CHLOROFORM-INDUCED SOLUBILIZATION

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Summary

1. Isolation of ATPase from rat liver submitochondrial particles by chloroform treatment requires the presence of ATP or ADP during enzyme solubilization. In the absence of adenine nucleotides the enzyme activity is very low although all protein components of F_1-ATPase are released. The low concentrations of ATP or ADP required (5 μM) indicate that the high affinity nucleotide-binding sites are involved in enzyme stabilization. Other nucleotides tested (ITP, GTP, UTP, CTP) were found to be less effective.

2. Polyacrylamide gel electrophoresis and immunodiffusion in agar plates revealed that in the absence of adenine nucleotides a fraction of F_1-ATPase released by chloroform treatment is split into fragments. The part of the dissociated enzyme molecule has a molecular weight identical with that of a β-subunit of F_1-ATPase.

3. Dissociation of the F_1-ATPase molecule could also be prevented by aurovertin.

4. Crude F_1-ATPase solubilized by chloroform treatment can be further purified by Sepharose 6B gel filtration. Specific ATPase activity of the purified enzyme was 90 μmol P_i/min per mg protein and the enzyme was composed of five protein subunits (α, β, γ, δ, ϵ) with molecular weights 58 000, 55 000, 28 000, 13 000 and 8000, respectively.

5. Chloroform-released F_1-ATPase from rat liver mitochondria displayed immunochemical cross-reactivity with that isolated from beef heart mitochondria.

Abbreviations: ATPase, adenosine triphosphatase (EC 3.6.1.3); F_1-ATPase, soluble ATPase from mitochondria; P_i, inorganic orthophosphate; SDS, sodium dodecyl sulphate.

Introduction

Mitochondrial ATPase (EC 3.6.1.3) can be easily released from beef heart submitochondrial particles by chloroform treatment [1] and purified by ion-exchange chromatography [2,3] or gel filtration [4]. According to the subunit composition and other enzyme properties, this purified ATPase preparation is identical with mitochondrial soluble ATPase (F_1-ATPase) isolated by other, more complicated methods (see Refs. 3 and 4).

Chloroform treatment when applied to rat liver submitochondrial particles, contrary to those from beef heart, solubilizes the protein fraction with only negligible ATPase activity [5]. However, an active F_1-ATPase preparation was solubilized from yeast submitochondrial particles when the original procedure was modified and the particles were preincubated with ATP prior to chloroform treatment [6]. This is in accordance with the known protective effect of ATP and other nucleotides against various treatments which inactivate the mitochondrial and chloroplast F_1-ATPase molecules [7—10].

In the present study an attempt was made to characterize the changes in the F_1-ATPase molecule accompanying the enzyme inactivation during the solubilization procedure. It was found that the main portion of the rat liver F_1-ATPase molecule was dissociated, and that the addition of various nucleotides before chloroform treatment prevented dissociation of the enzyme and preserved its integrity. The data are presented, which indicate that high affinity nucleotide-binding sites are involved in the stabilization of F_1-ATPase.

Material and Methods

Mitochondria were isolated from beef heart or rat liver according to published procedures [11,12]. Sonic submitochondrial particles were prepared as described earlier [4]. Particles were washed once with 0.25 M sucrose, 10 mM Tris/sulphate, 2 mM EDTA, pH 7.6, and suspended in the same medium to a protein concentration 20—30 mg/ml. The particles were stored at —20°C.

Crude F_1-ATPase was solubilized from mitochondrial membranes by chloroform extraction according to Beechey et al. [1]. Thawed rat liver or beef heart submitochondrial particles suspended in the sucrose/Tris/EDTA medium (2—20 mg protein/ml) were shaken with 0.5 vol. of chloroform for 20 s at room temperature. The aqueous phase containing solubilized F_1-ATPase was immediately separated by low speed centrifugation. Remaining insoluble material was removed from the water phase by centrifugation at 100 000 × *g* for 30 min at 20°C.

ATPase activity was measured in the medium (1.0 ml) containing 50 mM Tris/sulphate, 3 mM $MgCl_2$, 5 mM ATP, pH 8.4. Reaction was started by the addition of enzyme. After 3 min incubation at 30°C the reaction was stopped by the addition of 0.25 ml of 20% cold perchlorid acid. Samples were centrifuged and aliquots from the supernatant were taken for inorganic ortophosphate determination [13].

Electrophoresis was performed either on 10% (w/v) polyacrylamide gels containing 0.1% SDS [14], or on 7% (w/v) gels in the absence of SDS [15]. Gels were stained for protein [14] or for ATPase activity [16] and scanned at 550

nm. The following proteins were used as standards for molecular weights determination: cytochrome *c* (12 400), myoglobin (17 800), chymotrypsinogen A (25 000), glycerol-3-phosphate dehydrogenase (36 000), egg albumin (45 000), and bovine serum albumin (67 000).

Protein was determined according to Lowry et al. [17] with bovine serum albumin as standard.

Antiserum against beef heart chloroform-released F_1-ATPase purified on Sepharose 6B [4] was developed in mice. Each animal obtained 70 μg of enzyme protein mixed with Freund's complete adjuvant (1 : 1). Two 0.1 ml doses were applied subcutaneously on the neck and a 0.3 ml dose intraperitoneally. Booster injections of 130 μg enzyme without adjuvant were applied intraperitoneally on the 3rd and 5th week after the first immunization. Serum collected from animals killed 7 days after the last injection was used.

The double-diffusion test according to Ouchterlony [18] was carried out on plates containing 1.5% agarose in 20 mM Tris/sulphate, 0.05% azide, pH 7.4. The antiserum reacted with antigens at 20°C for 2 days, after which unprecipitated material was eluted by washing in 0.9% NaCl and precipitin lines were stained with Coomassie brilliant blue R 250 [19].

Results

Dissociation of liver mitochondrial F_1-ATPase during chloroform treatment

When frozen-thawed beef heart and rat liver submitochondrial particles were treated by chloroform according to Beechey et al. [1], approximately 10% of the membrane proteins was released from both types of particles. However, the ATPase activities of the protein fractions solubilized from the two sources differed considerably. Whereas the specific activity of beef heart enzyme preparation was 27 ± 6 and varied in a range of 17—36 μmol P_i/min per mg protein (11 experiments), the specific activity of the liver preparation was 7 ± 5 and varied in a range of 1—11 μmol P_i/min per mg (ten experiments).

SDS-polyacrylamide gel electrophoresis revealed, that even in the liver preparations exhibiting the lowest ATPase activities, polypeptides with molecular weights of 58 000 and 55 000 represented the major protein components (Fig. 1, trace 1), as in beef heart preparation [1].

When polyacrylamide gel electrophoresis of the liver preparation was performed in the absence of SDS, two protein bands, with R_F = 0.22 and R_F = 0.91, appeared (Fig. 1, trace 4). Only the protein band with the lower mobility (R_F = 0.22) could be stained for ATPase activity (Fig. 1, trace 6). The mobility of this band corresponded to the beef heart chloroform-released ATPase migrating under the same experimental conditions as a single protein band [1].

These results indicate that chloroform treatment of submitochondrial particles is quite efficient for the solubilization of F_1-ATPase protein components from the liver mitochondrial membrane; however, in contrast to beef heart, a portion of the liver F_1-ATPase is dissociated during chloroform extraction. Dissociation of the enzyme leads to its inactivation.

The second protein band that appeared in liver preparation (R_F = 0.91) was eluted from the gels and analyzed further by SDS-polyacrylamide gel electrophoresis. It was found that this band is formed by a single polypeptide with

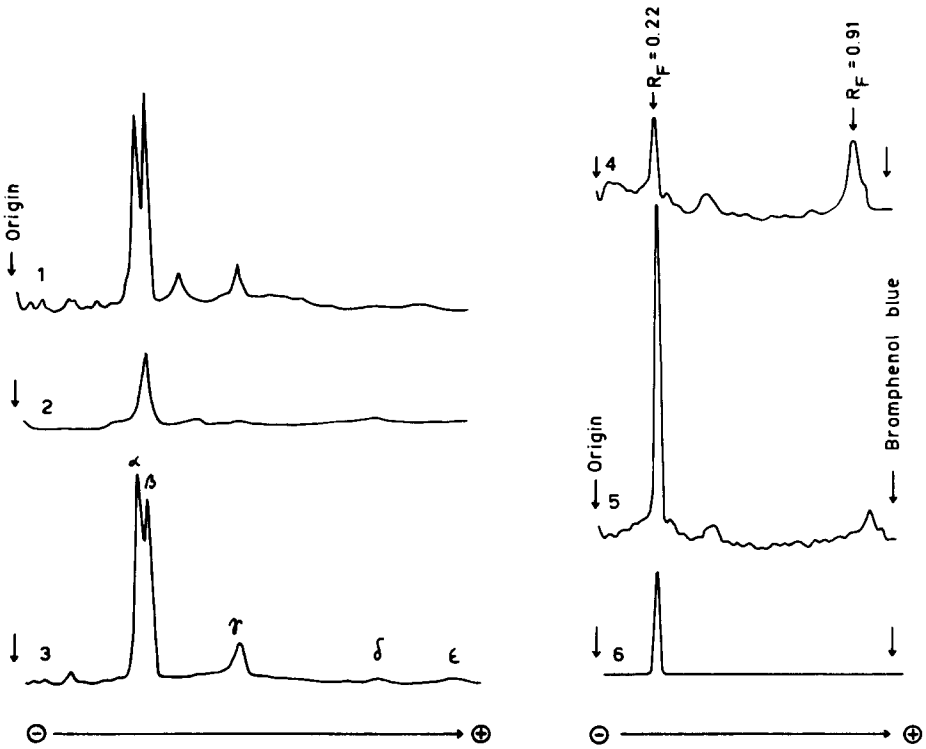

Fig. 1. Polyacrylamide gel electrophoresis of chloroform-released ATPase. The left side (traces 1—3), electrophoresis on 10% gels in the presence of SDS. The right side (traces 4—6), electrophoresis on 7% gels in the absence of SDS. Traces 1, 4 and 6, 10 μg of ATPase released by chloroform in the absence of ATP. Trace 2, electrophoresis of 100 μg of chloroform-released ATPase was performed on three 7% gels. The parts of gels corresponding to the position of the protein band with R_F = 0.91 were dissected, extracted and concentrated according to Weber and Osborn [14] and analysed on 10% gel containing SDS. Trace 3, chloroform-released ATPase purified on a Sepharose 6B column (5 μg, see Table IV). Trace 5, ATPase released by chloroform in the presence of ATP (10 μg). Gels were stained for protein (traces 1—5) and for ATPase activity (trace 6) and scanned at 550 nM.

molecular weight of 55 000, which comigrates with β-subunit of F_1-ATPase (Fig. 1, trace 2).

Protection of liver F_1-ATPase during chloroform treatment by nucleotides and aurovertin

Nucleotides, especially in the presence of EDTA, are known to protect mitochondrial and chloroplast F_1-ATPase against inactivation by various treatments [7—10]. Therefore in further experiments the effects of ATP and EDTA on the activity of crude chloroform-released F_1-ATPase from rat liver submitochondrial particles were assayed. ATP and/or EDTA were added to frozen-thawed submitochondrial particles and chloroform extraction was performed. As shown in Table IA, chloroform treatment of submitochondrial particles preincubated with ATP yielded a preparation with considerably higher ATPase activity. This effect of ATP was further supported by EDTA. In this case, specific ATPase activity was 3—5 times higher than in the absence of ATP and

TABLE I

PROTECTION OF F_1-ATPase ACTIVITY DURING CHLOROFORM-INDUCED RELEASE BY ATP AND EDTA

(A) Frozen-thawed particles prepared from frozen-thawed rat liver mitochondria in the absence of EDTA. (B) Fresh particles isolated from intact mitochondria. Suspension of particles (2 mg/ml) was preincubated for 2 min with 2 mM EDTA and/or 0.5 mM ATP before chloroform treatment. Specific ATPase activities of frozen-thawed and fresh particles were 4.0 and 5.1 μmol P_i/min per mg, respectively.

Particles	Additions	Chloroform-released ATPase		
		Protein (mg/ml)	Spec. act. (μmol P_i/min per mg)	Rel. act.
A	—	0.29	1.6	1.0
	EDTA	0.20	1.8	1.1
	ATP	0.22	5.2	3.3
	EDTA + ATP	0.21	7.7	4.8
B	EDTA	0.21	15.0	1.0
	EDTA + ATP	0.20	25.0	1.7

varied between 4 and 20 μmol P_i/min per mg (11 experiments). EDTA alone had no protective effect.

Specific ATPase activity of the chloroform-released protein fraction could be further increased, up to 21—28 μmol P_i/min per mg protein, when freshly prepared submitochondrial particles were used (Table IB). These values of enzyme activity are very similar to those obtained with frozen-thawed beef heart submitochondrial particles [1—4]. As shown in Table IB, the ATPase activity of the enzyme solubilized by chloroform treatment in the absence of

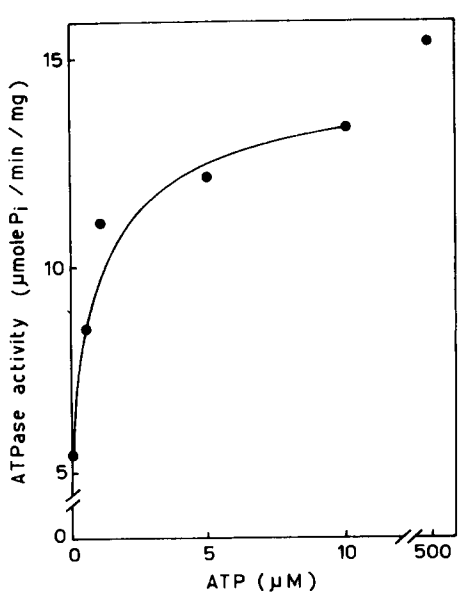

Fig. 2. The effect of ATP on specific activity of chloroform-released ATPase. ATP was added 2 min before chloroform treatment in the final concentrations 0.5—500 μM to the suspension of submitochondrial particles (2 mg/ml).

ATP was also increased. The relative protective effect of ATP in this case was lower. On the basis of these results, frozen-thawed liver particles were used as a model for the analysis of the nucleotide effect, and freshly prepared particles were used for isolation and purification of the liver F_1-ATPase.

As shown in Fig. 2, the protective effect of ATP can be observed already at μM concentrations. The ATPase activity was increased by 50% at 0.5—2.0 μM ATP. ADP was as potent as ATP in stabilization of F_1-ATPase molecule (Table II), and the protective effect appeared in the same concentration range as that of ATP (not shown).

Since the inactivation during chloroform treatment is connected with enzyme dissociation (Fig. 1, trace 4), it may be suggested that the protective effect of ATP is due to stabilization of the quaternary structure of F_1-ATPase. This was confirmed when the enzyme solubilized in the presence of ATP and EDTA was analyzed on a polyacrylamide gel. Most of the solubilized proteins were detected in the ATPase protein band ($R_F = 0.22$), while the other band with higher electrophoretic mobility ($R_F = 0.91$), containing a polypeptide with a molecular weight of 55 000, decreased (Fig. 1, trace 5).

The stimulating effects of various nucleotide triphosphates tested increased in the sequence: CTP < UTP < GTP < ITP < ATP (Table II). Polyacrylamide gel electrophoresis in the absence of SDS revealed that the enhancement of chloroform-released ATPase activity was always accompanied by an increase in the band of undissociated ATPase ($R_F = 0.22$) and a concomitant decrease of the protein band with higher electrophoretic mobility ($R_F = 0.91$). Stabilization of the enzyme was also accompanied by a decrease in the amount of protein remaining on the top of gels (not shown); therefore, the total amount of proteins that entered the gels increased (see Table II).

Similarly to the nucleotide effect, aurovertin was found as a stabilizing factor preserving F_1-ATPase integrity during chloroform extraction from rat liver mitochondrial particles. The protein content in the band corresponding the undissociated F_1-ATPase ($R_F = 0.22$) was significantly higher when the mem-

TABLE II

STABILIZATION OF F_1-ATPase DURING CHLOROFORM-INDUCED RELEASE BY VARIOUS NUCLEOTIDES

Nucleotides were added 2 min before chloroform treatment. Electrophoresis of chloroform-released ATPase was performed in the absence of SDS (20 μg protein/tube). Gels were stained for protein, scanned at 550 nm and areas of both protein bands of $R_F = 0.22$ and $R_F = 0.91$ were expressed in arbitrary units.

Nucleotide (0.5 mM)	Spec. ATPase act. (μmol P_i/min per mg)	Rel. act.	Areas of protein bands with $R_F = 0.22$ and $R_F = 0.91$		
			A	B	A/B
—	2.8	1.0	20	18	1.1
CTP	4.2	1.5	24	19	1.2
UTP	5.2	1.9	32	17	1.8
GTP	5.5	2.0	46	8	5.9
ITP	6.3	2.3	50	8	6.2
ADP	10.0	3.6	90	5	18.0
ATP	10.5	3.8	100	4	25.0

TABLE III

STABILIZATION OF F_1-ATPase DURING CHLOROFORM-INDUCED RELEASE BY AUROVERTIN

ATP or aurovertin were added 5 min before chloroform treatment to twice frozen-thawed rat liver submitochondrial particles suspended in a sucrose/Tris/EDTA medium (5 mg/0.2 ml). Electrophoresis on polyacrylamide gels and evaluation of the area of protein bands with R_F = 0.22 were performed as described in Table II.

Additions (mM)	Chloroform-released ATPase		
	Spec. act. (μmol P_i/min per mg)	Rel. act.	Area of protein band with R_F = 0.22
—	0.54	1.0	4.2
ATP (0.500)	8.70	16.3	100.0
Aurovertin (0.020)	1.00	1.9	52.0

brane particles were preincubated with aurovertin before chloroform extraction (Table III). In these experiments an aurovertin concentration similar to that already known to inhibit enzyme activity was used [20].

Crude F_1-ATPase released by chloroform from rat liver and beef heart submitochondrial particles was also analyzed by a double-diffusion test on agar plates using mouse antiserum developed against purified beef heart F_1-ATPase (Fig. 3). A single precipitin line was formed with proteins solubilized by chloro-

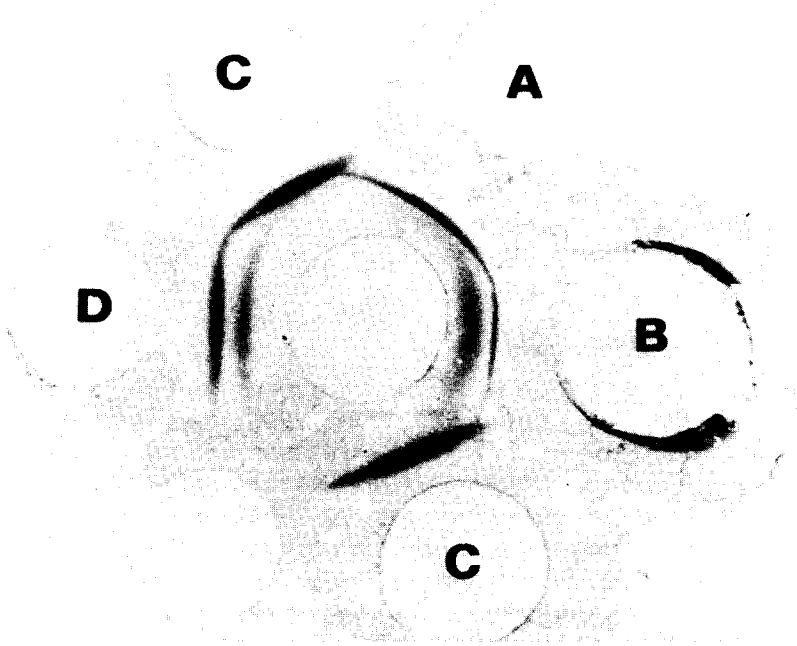

Fig. 3. Immunochemical reactivity of chloroform-released ATPases from beef heart and rat liver mitochondria. Antigens reacted with mouse antiserum developed against beef heart F_1-ATPase in double-diffusion test. Central well, antiserum (30 μl); A, liver ATPase stabilized during chloroform extraction by EDTA + ATP (14 μg); B, liver ATPase solubilized in the absence of ATP (34 μg); C. heart chloroform-released ATPase (8 μg); D, heart chloroform-released ATPase inactivated by freezing for a week at —20°C (13 μg).

form treatment of liver submitochondrial particles in the presence of ATP and EDTA (Fig. 3A), whereas two lines were detected when the enzyme was solubilized in the absence of ATP (Fig. 3B). Crude F_1-ATPase released from beef heart submitochondrial particles, representing homogenous antigen, produced a single precipitin line (Fig. 3C), whereas two lines were observed (Fig. 3D) when the heart enzyme was inactivated and dissociated by freezing [21]. Immunochemical cross-reactivity of rat liver and beef heart F_1-ATPases was also demonstrated (see Fig. 3A and C).

Purification of crude F_1-ATPase

As it has been mentioned above, crude chloroform-released F_1-ATPase of beef heart mitochondria can be further purified to homogeneity by Sepharose 6B gel filtration [4]. Similarly F_1-ATPase can be purified from membrane proteins solubilized from rat liver mitochondria (Fig. 4). The enzyme activity was eluted in a single peak of $K_{av} = 0.38$, which contained about 70% of proteins applied on the column. Specific activity of pooled fractions exhibiting ATPase activity was 35 μmol P_i/min per mg protein which was increased to 90 μmol P_i/min per mg protein when determined in the presence of HCO_3^- (Table IV). The purity of chloroform-released and Sepharose 6B-purified F_1-ATPase was demonstrated by SDS-polyacrylamide gel electrophoresis (Fig. 1, trace 3). The purified enzyme was composed of five polypeptides (α, β, γ, δ, ϵ) of molecular weights 58 000, 55 000, 28 000, 13 000 and 8000, respectively. Purification of chloroform-released ATPase thus results in an enzyme preparation quite comparable with those isolated from rat liver mitochondria by other procedures (see Ref. 5).

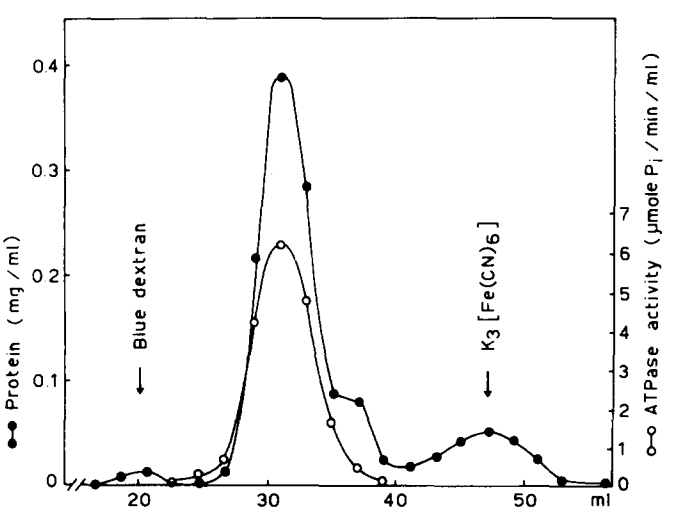

Fig. 4. Purification of chloroform-released ATPase on Sepharose 6B column. The enzyme was released from liver submitochondrial particles as described in Table IV and 2.3 mg of solubilized proteins in 1.25 ml were applied on a Sepharose 6B column (1.5 × 31 cm). The column was equilibrated and eluted with a medium containing 50 mM Tris/sulphate, 1 mM ATP, 1 mM EDTA, pH 7.4, at room temperature. The flow rate was 10 ml/h and 2-ml fractions were collected for protein and ATPase activity determinations.

TABLE IV

ISOLATION AND PURIFICATION OF F_1-ATPase FROM RAT LIVER MITOCHONDRIA

ATPase was released from feshly prepared submitochondrial particles (23 mg/ml) in the presence of 1 mM ATP and purified on Sepharose 6B column (see Fig. 4). In the case of Sepharose 6B effluent all fractions exhibiting ATPase activity were combined.

Fraction	Protein (%)	Spec. act. (μmol P_i/min per mg)	Total activity (%)
Particles	100.0	5.5	100.0
Chloroform-released ATPase	9.0	25.0	40.9
Sepharose 6B effluent	6.0	35.7	38.9
		89.3 *	69.6 *

* Measured in the presence of 30 mM $KHCO_3$.

Discussion

F_1-ATPase isolated from mitochondria or other types of energy-transducing membranes is a complex molecule composed of five different polypeptides. The enzyme molecule is formed by dimeric or trimeric structure of two major subunits (α and β) [22,23], which are necessary for the enzyme hydrolytic activity [24] and are complemented by three other polypeptides (γ, δ and ε) whose function is not yet well defined. Various factors have been described that induce dissociation of the enzyme and its inactivation [21,22,25—27] and the β-subunit has been identified as the dissociated fragment [22,25,26].

Also in our experiments, the inactivation of the rat liver F_1-ATPase during chloroform treatment was accompanied by the appearance of a dissociated polypeptide displaying the same electrophoretic mobility as β-subunit of F_1-ATPase, indicating that the enzyme inactivation is accompanied by the dissociation of the β-subunit. The other fragments of the enzyme molecule could not be identified because these proteins aggregate and do not enter the polyacrylamide gels. An unassembled β-subunit was also identified during purification of F_1-ATPase released by chloroform treatment from yeast submitochondrial particles [28].

Our results have shown that the dissociation of rat liver F_1-ATPase and the concomitant loss of the enzyme activity can be prevented by adenine nucleotides. Addition of adenine nucleotides is not required when the enzyme is isolated by the same experimental procedure from beef heart mitochondria. This indicates different stability of beef heart and rat liver F_1-ATPases. Low stability of the liver enzyme during solubilization as well as the inactivation of membrane-bound ATPase during freezing-thawing (see Table I) may result either from different quarternary structures of rat liver and beef heart F_1-ATPase or from different amounts of endogenous stabilizing factor in these two enzymes. Such a factor could be the endogenous protein inhibitor of mitochondrial ATPase [29,30] or endogenous adenine nucleotides [6—10].

Mitochondrial F_1-ATPase is known to contain two types of binding sites for adenine nucleotides. These differ both in affinity to nucleotides and in the rate of exchange between bound and free ligands. Low affinity binding sites appear

to be localized in the catalytic center of the F_1-ATPase, whereas the role of high affinity binding sites is not yet completely clarified [31]. As the stabilizing effect of adenine nucleotides occured at a μM concentration range, it seems that high affinity binding sites are involved in the enzyme stabilization. The affinity of this type of binding sites for ADP on rat liver F_1-ATPase (K_D = 0.9—1.3 μM), described by Pedersen [32], is in good agreement with this assumption. The stabilizing effects of various nucleotide triphosphates decreased in the sequence: ATP > ITP > GTP > UTP > CTP. This indicates that the nucleotide specificity of high affinity binding sites involved in stabilization of F_1-ATPase molecule is the same as the specificity of binding sites in the catalytic center of F_1-ATPase [33].

The stabilizing effect of aurovertin on the F_1-ATPase molecule during chloroform treatment, though less pronounced than that of adenine nucleotides, indicates that the binding of this ligand also renders the enzyme molecule more resistant to the chloroform-induced dissociation. Moreover, since both the aurovertin and the nucleotide binding sites are localized on the β-subunit of F_1-ATPase [22,28,34,35] it may be supposed that the interaction of this subunit with a specific ligand induces a conformational change that protects the enzyme molecule against dissociation.

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