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A SIMPLE PROCEDURE FOR ISOLATING ADENOSINE TRIPHOSPHATASE FROM MITOCHONDRIA

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Summary

A simple method for isolation of adenosine triphosphatase (EC 3.6.1.3) from mitochondria is described. The enzyme is released from mitochondrial Lubrol particles by drastic sonication and purified by gel filtration on Sepharose 6-B. The described procedure is effective in isolating adenosine triphosphatase from rat liver as it is from beef heart mitochondria. The enzyme isolated from beef heart has a specific activity of 120 μmol P/min per mg protein and enzyme isolated from rat liver has a specific activity of 70 μmol P/min per mg protein when measured as a release of inorganic phosphate.

Several methods have been described for isolating mitochondrial cold-labile oligomycin-insensitive adenosine triphosphatase (F_1-ATPase) (EC 3.6.1.3) from mammalian tissues [1—6]. As a rule, ATPase is solubilized from frozen mitochondria or from sonicated membrane particles by drastic sonication. The enzyme is further purified by ammonium sulfate or protamine sulfate precipitation combined with ion exchange chromatography and gel filtration. These rather complicated and time-consuming techniques produce sufficiently homogenous enzyme preparation, but differ in yield and are usually specific for one type of tissue. ATPase can be also released by chloroform extraction of submitochondrial particles from beef heart and possibly from some other tissues; however, the enzyme is less pure [7]. An aim of this study was to prepare a simple procedure for isolation of pure F_1-ATPase with a high yield, applicable for different tissues.

The method presented is based on the finding that the non-ionic detergent Lubrol-WX selectively solubilizes about 70% of all mitochondrial proteins from rat liver mitochondria [8]; ATPase, however, remains attached to the membrane fragments. Hence, with respect to ATPase, Lubrol particles represent the highly enriched inner membrane preparation. From these particles,

ATPase is solubilized by drastic sonication in a 100% yield and the enzyme is separated from the other soluble proteins by gel filtration on Sepharose 6-B. This procedure is as effective in isolating F_1-ATPase from rat liver as it is from beef heart mitochondria. The isolated enzyme is highly purified when controlled by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, and has a specific activity up to 120 μmol P/min per mg protein for beef heart preparation and up to 70 μmol P for liver preparation when assayed as a release of inorganic phosphate.

Preparation of Lubrol particles. Lubrol particles were prepared by the modified method of Chan et al. [8]. Frozen beef heart mitochondria (300—400 mg of protein) isolated according to Smith [9] or rat liver mitochondria isolated according to Schneider and Hogeboom [10] were suspended in 0.1 M sucrose, 10 mM Tris sulfate, 1 mM EDTA, pH 7.4 (STE medium) at a protein concentration of 25—30 mg/ml. Unless otherwise stated, the temperature during isolation procedure was kept at 25°C. A solution of Lubrol-WX (19 mg/ml) was added drop-wise to the mixed mitochondrial suspension. When the Lubrol concentration reached 0.15 mg/mg protein, the suspension was mixed for another 3 min, then diluted 3—5 times by the STE medium and centrifuged for 30 min at 100 000 × *g*. The sediment containing Lubrol particles was suspended in an STE medium containing 4 mM ATP.

Solubilization of ATPase by drastic sonication. The suspension of Lubrol particles was alcalinized by an ammonia solution (1:5) to pH 9 and mixed for 30 min. The pH was adjusted to 8 and the mixture of particles was sonicated in 10—15 ml portions for 20 min. An ultrasonic desintegrator MSE-100 W with a 19-mm pestle was used at maximum output. During sonication, the temperature slowly rose to 50°C. The sonicated suspension was then cooled to 30°C and centrifuged for 30 min at 100 000 × *g*. Supernatant was collected.

Gel filtration of solubilized proteins. Solubilized proteins of 20—60 mg in 3—6 ml were applied to a column of Sepharose 6-B (80 × 2 cm), equilibrated with a solution containing 50 mM Tris sulfate, 1 mM EDTA, 2 mM ATP, pH 7.4. Proteins were eluted by the same solution. The flow of effluent was maintained at 10 ml/h and 5 ml portions were collected for protein [11] and ATPase determination. ATPase activity was determined as a release of inorganic phosphate [12] or spectrophotometrically in the presence of an ATP-regenerating system as described by Pullman et al. [13].

Proteins were eluted from the column in 4—5 peaks. The first one, corresponding to the void volume, evidently represented the remaining particle proteins (Fig. 1). Almost all ATPase activity emerges as a single zone corresponding to the third protein peak behind the void volume (K_{av} = 0.36 − 0.37) (Fig. 1).

Table I demonstrates that through this isolation procedure F_1-ATPase can be purified either from beef heart or from rat liver mitochondria. ATPase isolated from beef heart mitochondria represents 6% of the original mitochondrial proteins and has a specific activity 50.9 μmol P/min per mg protein. Specific activity values in individual experiments varied between 50—80 μmol P/min per mg protein (activity measured at pH 7.4). During isolation, specific activity of the purified enzyme increases 7-fold, apparently due to the removal of protein inhibitor [14].

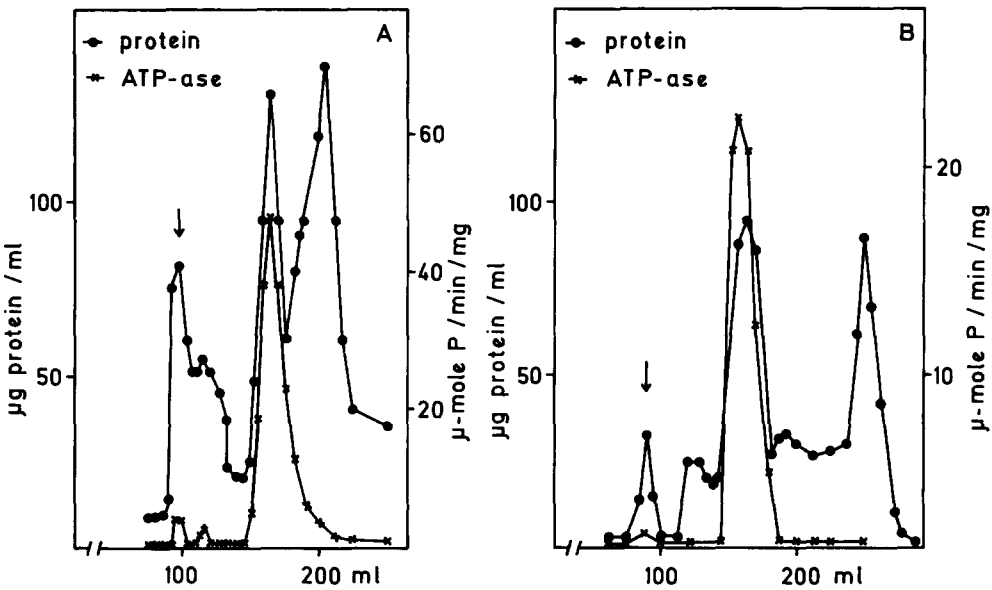

Fig. 1. Gel filtration of solubilized proteins on Sepharose 6-B. A, beef heart; B, rat liver. For enzyme activity determination 20—100 µl of effluent were used. Enzyme activity was determined as described in Table I. Arrow indicates elution position of blue dextran.

TABLE I

PURIFICATION OF MITOCHONDRIAL ATPase

Frozen mitochondria were used for ATPase isolation. Enzyme activity was measured as the release of inorganic phsophate. The incubation mixture contained 50 mM KCl, 10 mM Tris sulfate, 3 mM $MgCl_2$, 5 mM ÁTP (pH 7.4), 0.2—0.5 mg of mitochondria or Lubrol particles, 0.05 mg of solubilized proteins and 0.005 mg of ATPase per 2 ml. The enzyme activity was followed for 3 min at 30°C. Specific activity is expressed as µmol P/min per mg protein. Total activity is expressed in percent of original mitochondrial total activity.

Step	Beef heart			Rat liver		
	Protein (%)	Spec. act.	Total act.	Protein (%)	Spec. act.	Total act.
Mitochondria	100	0.44	100	100	0.31	100
Lubrol particles	80	0.88	175	44	0.79	112
Solubilized proteins	18	2.50	102	13	2.10	89
ATPase	6	50.90	694	1.3	25.40	108

TABLE II

SPECIFIC ACTIVITY OF F_1-ATPase FROM BEEF HEART AND RAT LIVER MITOCHONDRIA

Enzyme activity was measured as described in Table I. *Indicates parallel determination of enzyme activity by the spectrophotometric method. In this case, 0.2 µg of protein per 2.7 ml was used.

Experimental conditions	Spec. act. (µmol P/min per mg protein)	
	Beef heart ATPase	Rat liver ATPase
pH 7.4	55.0	29.8
pH 8.2	84.6 (168.2)*	44.4 (68.5)*
pH 8.2, 30 mM $KHCO_3$	122.3	78.4
pH 8.2, 24 h at 20°C	45.8	32.2
pH 8.2, 30 mM $KHCO_3$, 24 h at 20°C	70.0	50.0

ATPase isolated from rat liver mitochondria (Table I) represents 1.3% of the original mitochondrial proteins, and has a specific activity of 25.4 μmol P/min per mg protein. Values in individual experiments varied between 20—35 μmol P/min per mg protein. Specific activity increases 80-fold during isolation, and 108% of the total original enzyme activity is recovered (Table I).

Specific activity of purified ATPase can be further increased when measured at a higher pH, or in the presence of bicarbonate ions (Table II), as was shown with other F_1-ATPase preparations [5,6,15]. In these conditions, values as high as 122 μmol P/min per mg for beef heart ATPase and 78 μmol P/min per mg for liver ATPase can be obtained. When the spectrophotometric assay is used, the values of specific activity of 160 μmol P/min per mg for beef heart ATPase and of 68 μmol P/min per mg for liver ATPase can be obtained, even in the absence of bicarbonate ions (Table II).

Properties of isolated F_1-ATPase. ATPase isolated from beef heart as well as from rat liver mitochondria is oligomycin insensitive, cold-labile, and stable at room temperature in the presence of 2 mM ATP at least for 48 h without

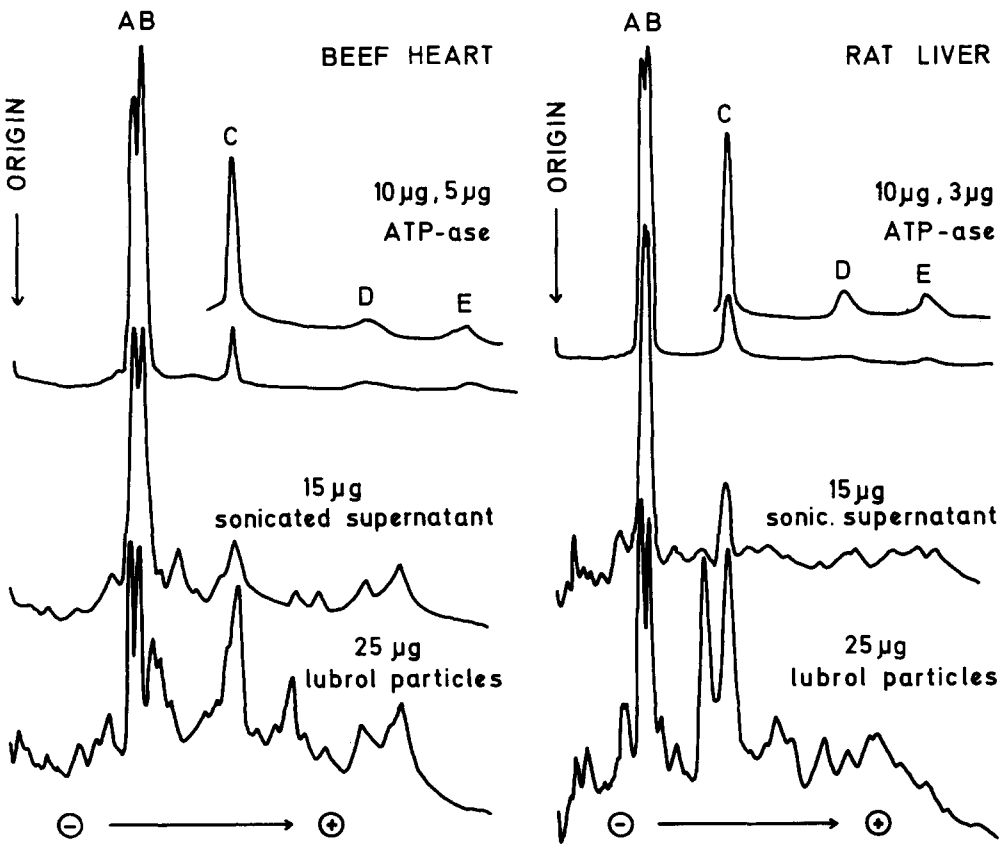

Fig. 2. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Samples of isolated ATPase, of soluble proteins released by sonication from Lubrol particles (sonicated supernatant) and of Lubrol particles were dissociated by 3 min incubation at 100°C in the presence of 1% sodium dodecyl sulfate and 2% mercaptoethanol. Gels, stained with Coomassie brilliant blue (R 250) were scanned at 550 nm at the speed 1.5 cm/min in an Beckman ACTA III spectrophotometer equipped with gel scanner attachment.

loss of enzyme activity. Sedimentation velocity analysis (Spinco analytical centrifuge, model E, 3.2 mg of enzyme per ml of 0.05 M Tris sulfate, 1 mM EDTA, pH 7.4) proved that isolated ATPase sediments as a single band. Repeated gel filtration also showed that the enzyme is homogenous. The molecular weight of both beef heart and rat liver ATPases determined by gel filtration were found to be similar with F_1-ATPase preparations isolated by other methods. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate [16] of rat liver and beef heart enzyme preparations showed equal protein band distribution (Fig. 2), typical for F_1-ATPase [1,2,4]. Three major protein bands (A, B, C) reflect approximative molecular weights of 53 000, 52 000 and 30 000, respectively. Two other minor protein bands (D, E) of molecular weights 13 000 and 9000 are evident, if a larger amount of protein is used for electrophoresis (Fig. 2).

TABLE III

RECONSTITUTION OF OLIGOMYCIN SENSITIVE ATPase

NaBr-particles prepared according to MacLennan et al. [17] and F_1-ATPase were suspended in an STE medium (1 ml) and incubated for 30 min at 25°C. ATPase was assayed as the release of inorganic phosphate. 3 μg/ml of oligomycin were used. The ATPase activity of NaBr-particles was lower than 0.015 μmol P/min per mg.

	ATPase activity (μmol P/min)	Inhibition by oligomycin (%)	Oligomycin sensitive activity per mg of NaBr-particles
Beef heart F_1-ATPase (5 μg)	0.195	10.7	—
Beef heart F_1-ATPase and beef heart NaBr-particles (170 μg)	0.205	64.8	0.66
Beef heart F_1-ATPase and rat liver NaBr-particles (170 μg)	0.224	45.4	0.46
Rat liver F_1-ATPase (6 μg)	0.223	5.4	—
Rat liver F_1-ATPase and rat liver NaBr-particles (140 μg)	0.221	47.5	0.66
Rat liver F_1-ATPase and beef heart NaBr-particles (140 μg)	0.198	52.5	0.66

The isolated enzyme reassociates very easily with particles depleted of ATPase by treatment with 3 M NaBr (NaBr-particles) [17]. As shown in Table III, this reassociation restores the oligomycin sensitivity of the enzyme. Beef heart ATPase is equally effective in reassociating with beef heart as well as rat liver NaBr-particles. In the same way, rat liver ATPase is equally effective in reassociating with both types of NaBr-particles (Table III). The reassociated enzyme cannot be washed from the membrane particles by repeated 100 000 × *g* centrifugation.

From the data presented, it is evident that this procedure for isolating soluble mitochondrial adenosine triphosphatase can be used for beef heart and rat liver mitochondria. The procedure is also very effective for isolating ATPase from mitochondria of brown adipose tissue where the amount of the enzyme is extremely reduced [18]. This applicability for rat liver and brown fat is the advantage of the method in comparison with chloroform extraction method [7] which in our experiments released from beef heart submitochondrial particles 7% of particle protein and 150% of original ATPase activity, while from liver and brown fat submitochondrial particles, 10—17%

of particle protein and only 0.5—5% of ATPase activity. The method described therefore enables the easy isolation of pure ATPase with high specific activity from mitochondria of various tissues, with a high yield.

References

1 Horstmann, L.L. and Racker, E. (1970) J. Biol. Chem. 245, 1336—1344
2 Senior, A.E. and Brook, J.C. (1975) Arch. Biochem. Biophys. 140, 257—266
3 Kozlov, I.A. and Mikelsaar, H.N. (1974) FEBS Lett. 43, 212—214
4 Catteral, W.A. and Pedersen, P.L. (1971) J. Biol. Chem. 246, 4987—4994
5 Lambeth, D.O. and Lardy, H.A. (1971) Eur. J. Biochem. 22, 335—363
6 Selwyn, M.J. (1967) Biochem. J. 105, 279—288
7 Beechey, R.B., Hubbard, S.A., Linnet, P.E., Mitchell, D.A. and Munn, E.A. (1975) Biochem. J. 148, 533—537
8 Chan, T.L., Greenawalt, J.W. and Pedersen, P.L. (1970) J. Cell. Biol. 45, 335—363
9 Smith, A.L. (1967) in Methods in Enzymology (Estabrook, M.E. and Pullman, M.E., eds.) Vol. X, pp. 81—86, Academic Press, New York
10 Schneider, W.C. and Hogeboom, G.H. (1950) J. Biol. Chem. 183, 123—128
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
12 Lindberg, O. and Ernster, L. (1956) in Methods in Biochemical Analysis (Glick, D., ed.) pp. 1
13 Pullman, M.E., Penefsky, H.S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322—3329
14 Pullman, M.E., and Monroy, G.C. (1963) J. Biol. Chem. 238, 3762—3769
15 Pedersen, P.L. (1976) Biochem. Biophys. Res. Commun. 71, 1182—1188
16 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
17 MacLennan, D.H., Smoly, J.M. and Tzagaloff, A. (1968) J. Biol. Chem. 243, 1589—1597
18 Houštěk, J. and Drahota, Z. (1977) Biochim. Biophys. Acta, in the press