PURIFICATION OF A SOLUBLE ATPase OF RAT LIVER

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1. Introduction

The presence in mitochondria of a Mg^{2+} -dependent membrane ATPase [1–6] and a soluble ATPase activated by dinitrophenol (DNP) [7] has been demonstrated. We have found a soluble ATPase, not activated by DNP, in the soluble mitochondrial fraction. The purpose of this paper is to describe the purification and several properties of this ATPase.

2. Methods

2.1. *Preparation of the matrix*

The mitochondria were prepared according to Harel et al. [8]. The matrix was obtained by freezing and thawing mitochondria, followed by a 165,000 *g* centrifugation for 2 hr. Proteins were determined by the method of Lowry [9].

2.2. *Determination of ATPase activity*

For this assay, the incubation mixture contained, in a final vol of 3 ml, the following reagents : 150 mM K-phthalate-KOH buffer pH 5.8, 5 μM Na-ATP (K. Roth, Germany), and 0.05 ml of enzyme preparation. Incubation was continued for 10 min at 45°. The reaction was stopped by the addition of 0.1 ml of 100% trichloracetic acid and the incubation tubes were placed at 0°. The proteins were sedimented by centrifugation and P_i determined in the supernatant according to Briggs [10].

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2.3. *Polyacrylamide gel electrophoresis*

Polyacrylamide gel at 7% concentration, containing 0.1% SDS was prepared in 0.1 M phosphate buffer at pH 7.1 [11]. 20 μg samples, previously treated for 1 hr with 1% SDS, were applied on the gel columns. The reference standards were cytochrome *c*, myoglobin and chymotrypsinogen. Bromophenol was used to mark the front. After electrophoresis for 8 hr at 4 mA the gels were stained with Coomassie Brilliant Blue (0.05% in 10% acetic acid) for 12 hr.

3. Results

3.1. *Purification*

3.1.1. Ammonium sulphate precipitation

Sufficient solid ammonium sulphate to obtain 53% saturation was gradually added with stirring to the matrix. The resulting precipitate was removed by centrifugation. Ammonium sulphate was then added to the supernatant to obtain 85% saturation; after centrifugation, the precipitate was dissolved in phthalate buffer, pH 5.8.

3.1.2. Heating at 55°

The solution was heated for 15 min at 55°. The precipitate was sedimented by centrifugation. The supernatant was dialysed against phthalate buffer, pH 5.8 for 12 hr.

3.1.3. DEAE-cellulose chromatography

The dialysed solution was brought to pH 7.4 and applied to a 3 × 45 cm DEAE-cellulose (Serva, Heidelberg, Germany) column which had been equilibrated with 25 mM Tris-HCl buffer pH 7.4. After washing,

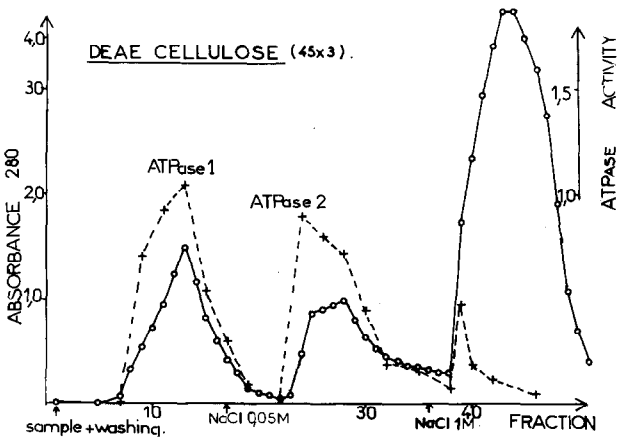

Fig. 1. DEAE-cellulose chromatography. The column was charged with 55 mg of protein of the ammonium sulphate fraction (53%–85%). The flow rate was approx. 40 ml/hr. 3.5 ml fractions were collected. The absorbance at 280 nm (o——o) was measured for each fraction and ATPase activity (x - - - x) was determined as described in Methods.

proteins were eluted with a discontinuous NaCl gradient. We obtained 2 peaks containing ATPase activity which we designated ATPase 1 and ATPase 2 (fig. 1).

3.1.4. Sephadex G-100 filtration

ATPase 1 and 2 were then purified separately. The samples were applied to a 3 X 110 cm Sephadex G-100 column equilibrated with phthalate buffer, pH 5.8. ATPase 1 was eluted in fractions 51–57 (fig. 2a) and ATPase 2 in fractions 26–32 (fig. 2b).

3.1.5. Hydroxylapatite chromatography

Fractions containing ATPase were pooled, precipitated with saturated ammonium sulphate and dialysed for 8 hr against 1 mM phosphate buffer, pH 6.8. The dialysed solutions were applied to a 0.8 X 10 cm hydroxylapatite column previously equilibrated with 1 mM phosphate buffer pH 6.8. After washing, the column was eluted with a discontinuous gradient of 1 mM, 10 mM, 50 mM and 300 mM phosphate buffer, pH 6.8 containing 3 M KCl. ATPases 1 and 2 were eluted at a phosphate buffer concentration of 10 mM. Results of purification procedures are shown in table 1. All the following enzymatic studies were performed with about 130-fold purified preparations.

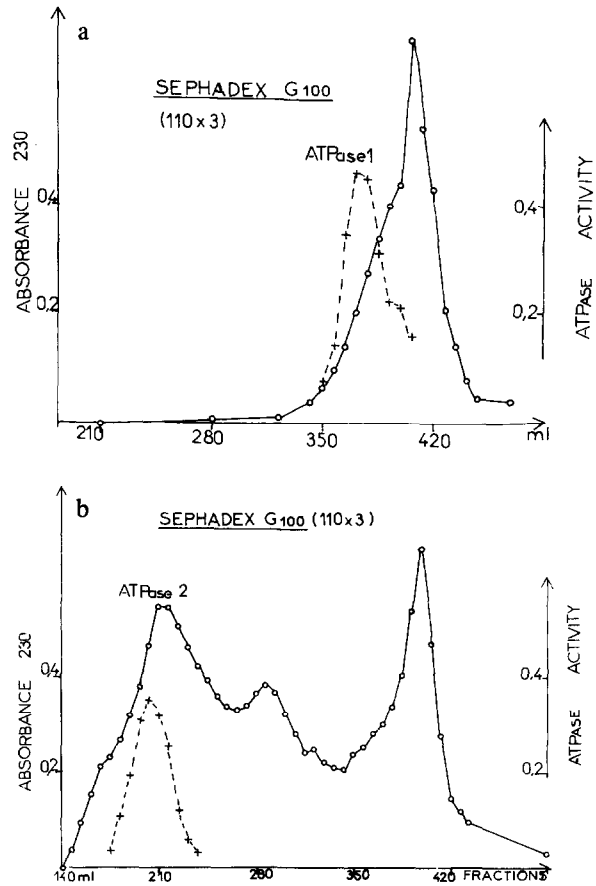

Fig. 2. Sephadex G-100 filtration. The column was charged with about 20 mg of protein of the DEAE-cellulose peak 1. The flow rate was approx. 30 ml/hr. 7 ml fractions were collected. The absorbance at 280 nm (o——o) was measured for each fraction and ATPase activity (x - - - x) was determined as described in Methods.

3.2. *Determination of the molecular weight of the two fractions containing ATPase activity*

3.2.1. Gel filtration on Sephadex G-100

The 2 purified fractions were filtered on Sephadex G-100 under the conditions previously described. Serum albumin, ovalbumin, trypsin, and cytochrome *c* were used as markers. We represent log molecular weight (M.W.) as a function of the elution volume (fig. 3). Under these conditions, we found the following M.W.: ATPase 1: 15,900 daltons; ATPase 2: 61,000 daltons.

3.2.2. Polyacrylamide gel electrophoresis

We first studied ATPase 1 by polyacrylamide gel

Table 1
Purification of ATPases.

Steps	Protein (mg)	Specific Activity (μM P_i/min/mg protein)	Purif. factor	Yield (%)
Mitochondria	18,000	0.075	1	–
Matrix	5,050	0.218	2.9	100
Ammonium sulphate precipitate	1,430	0.427	5.7	55.5
Heating	980	0.577	7.7	46.0
ATPase 1:				
DEAE-cellulose	110	1.44	19.3	14.5
Sephadex G-100	23	4.47	59.4	9.4
Hydroxylapatite	7.5	9.12	123.0	6.2
ATPase 2:				
DEAE-cellulose	92	2.07	27.6	17.3
Sephadex G-100	15.5	6.12	82.0	8.1
Hydroxylapatite	5.5	10.07	134	5.0

Experiments were performed with mitochondria from 120 rat livers.

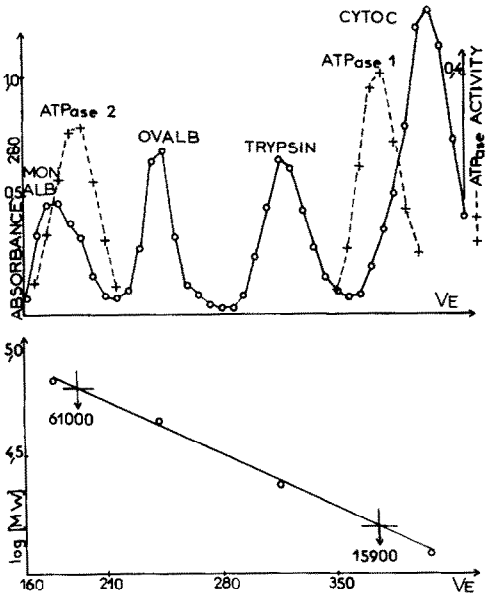

Fig. 3. Sephadex G-100 filtration. The column (3 × 110 cm) was charged with 1 mg of each enzymatic form and about 10 mg of albumin, ovalbumin and cytochrome *c*. The flow rate was approx. 30 ml/hr. 7 ml fractions were collected. The absorbance at 280 nm (o——o) was measured for each fraction and ATPase activity (x - - - x) was determined as described in Methods.

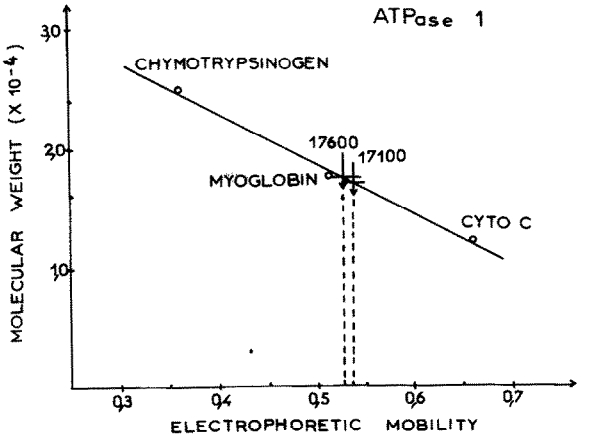

Fig. 4. Polyacrylamide gel electrophoresis was performed as described in Methods. The molecular weights were calculated by comparing the electrophoretic mobilities of the 2 bands to those of the 3 standard proteins.

electrophoresis. There were 2 bands very close together without any other contaminating proteins. The determination of the M.W. of these 2 proteins were made using cytochrome *c*, myoglobin and chymotrypsinogen as reference standards. The M.W. found for these 2 bands are 17,100 and 17,600 (fig. 4).

4. Discussion

We isolated a soluble ATPase which appears to exist in 2 molecular species. We have purified these 2 forms about 130-fold. The M.W. of the 2 electrophoretic bands obtained with ATPase 1 are 17,100 and 17,600; these values are very similar to the values found using Sephadex G-100 filtration (15,900). This latter method showed the presence of only one active compound. The question arises, what is the significance of these 2 bands? It may be that 1 band corresponds to ATPase and the other to a contaminating protein. Another possibility would be that the 2 bands correspond to 2 isoenzymes of this enzyme.

The M.W. of ATPase 2 is 61,000 and that of ATPase 1 is 15,900. Since their properties are very similar it seems possible that ATPase 2 is a tetramer form of ATPase 1.

The M.W. of a membrane ATPase isolated from *Streptococcus fasecalis* by Schnebli [12] was 385,000. Penefsky et al. [13] found for a bovine heart mitochondria ATPase a M.W. of 284,000. All these M.W. were higher than the value found for the soluble ATPase we isolated.

During the preparation of this paper, Kalf and Grèce [14] reported the existence of a soluble ATPase in rat liver mitochondria. They did not, however, show the existence of 2 enzymatic forms, nor did they give any indication of the M.W. They purified their preparation only about 10-fold. Our soluble ATPase differs from the Mg^{2+}, Ca^{2+}, and Na^{+}- K^{+} dependent ATPase. Addition of EDTA to the incubation mixture or careful elimination of Na and K are without influence on the ATPase activity.

The study of the properties of this enzyme will be published in a following paper.

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