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PURIFICATION OF A SOLUBLE ATPase FROM RAT LIVER MITOCHONDRIA BY AMP-SEPHAROSE AFFINITY CHROMATOGRAPHY

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Summary

ATPase (ATP phosphohydrolase, EC 3.6.1.3) activity was shown in the soluble fraction of rat liver mitochondria. Two molecular forms (ATPase 1 and 2) were isolated. ATPase 1 has already been studied. The present paper deals with the purification method of ATPase 2 which was achieved by the following steps: $(NH_4)_2SO_4$ precipitation, DEAE-cellulose chromatography, hydroxyapatite chromatography, Sephadex G100 filtration and AMP-Sepharose affinity chromatography.

The purified protein was characterized by bidimensional polyacrylamide gel electrophoresis.

Molecular weight evaluated by SDS-polyacrylamide gel electrophoresis and Sephadex G100 gel filtration was found to be 61 500 ± 3000.

Introduction

The presence of a Mg^{2+}-dependent ATPase (ATP phosphohydrolase, EC 1.6.1.3) has been demonstrated in mitochondria [1,2]. This enzyme catalyses the hydrolysis of ATP and also the reverse reaction. The enzyme complex is localized in the internal membrane of mitochondria. Several authors have been able to solubilize (from the Mg^{2+}-dependent ATPase) a protein called F1 factor which possesses an ATPase activity, but which has lost the property of catalysing the synthesis of ATP. The F1 factor is oligomycin-insensitive, is activated by magnesium and irreversibly cold-inactivated. The structure of this protein is complicated and has been recently described by several authors [3—6]. Other studies revealed proteins which had an ATPase activity in the soluble fraction of rat liver mitochondria [7—9]. Recently, we found an ATPase activity in the soluble fraction of rat liver mitochondria [10,11]. We showed that this activity corresponds to two molecular forms (ATPase 1 and ATPase 2). These two pro-

teins are distinct, sharing only one common property, that of the specific hydrolysis of nucleoside triphosphates. We observed in immunochemical and structural studies, that ATPase 1 is different from the subunits of the F1 factor. This paper describes the purification of an ATPase from rat liver mitochondria which we designate as ATPase 2. Its purification was achieved by a final AMP Sepharose affinity chromatography step.

Materials and Methods

Preparation of the soluble mitochondrial fraction

The technique was modified from that described by Harel et al. [12] to deal with large quantities of rat liver. This technique produced a mitochondrial preparation free of nuclei, cellular debris and cytoplasmic contamination. The homogenized mitochondrial pellet was immersed in liquid air and then allowed to thaw at room temperature. After thawing, the solution was centrifuged at 105 000 $\times g$ for 1 h. The supernatant contained the soluble mitochondrial fraction. Proteins were determined by the method of Lowry et al. [13] or, during the later stages of the purification, by the spectrophotometric method of Ehresmann et al. [14].

Determination of ATPase activity

The ATPase activity was measured by assay of liberated phosphate in an incubation medium which contained (final volume, 1.6 ml): 1.4 ml 10 mM cacodylate/HCl buffer (pH 6.0) and 0.1 ml enzyme preparation.

The incubation tubes were pre-incubated for 5 min and the reaction started by the addition of 0.1 ml 25 mM disodium ATP (final concentration 1.56 mM). The incubation was continued at 48°C for 10 min. The reaction was stopped by addition of 0.1 ml concentrated trichloroacetic acid and the tubes were then placed in an ice-water bath. As a control, the non-enzymatic hydrolysis of ATP was carried out in parallel during incubation; the amount of phosphate present in the enzymatic preparation was also measured. The liberated phosphate was measured directly using the method of Weil-Malherbe et al. [15] which was modified for very small quantities (0.1 μmol) as described by Le Deaut et al. [16].

Preparation of AMP-Sepharose

We prepared the N^6-(6-aminohexyl)-5′-AMP, using for starting materials the sodium salt of 6-mercaptoethanol ribose 5′-monophosphate and 1,6-hexane diamine according to the method of Craven et al. [17]. AMP-Sepharose was prepared by coupling the N^6-(6-aminohexyl)-5′-AMP to Sepharose 4B following the method of Axen et al. [18].

Electrophoresis on polyacrylamide gel

7.5% Polyacrylamide gels were prepared following the method of Maurer [19]. The protein preparation for analysis (10—50 μg) was dialyzed before use for 12 h against the electrophoresis buffer (Tris/glycine, pH 8.3 [50 mM Tris]-1 mM dithiothreitol. The current during electrophoresis was maintained at 1 mA/gel at 4°C. One gel was stained for 4 h with Coomassie blue (0.25%) in a mix-

ture of methanol/acetic acid/H_2O (50 : 10 : 40 v/v) and afterwards destained in a mixture of methanol/H_2O/acetic acid (5 : 87.5 : 7.5 v/v). A second gel was cut into 2 mm slices and the slices homogenized separately in 10 mM cacodylate/HCl buffer (pH 6.0) and left for 6 h in the solution. The pH of the homogenate was adjusted to 6.0 with 0.1 N HCl just prior to incubation. Polyacrylamide gradient gel slab electrophoresis were performed with an electrophoresis apparatus GE4 (Pharmacia, Uppsala, Sweden). We have also carried out electrophoresis in a double polyacrylamide gel system (8 and 10%) in the presence of 0.1% SDS.

Results

Purification of ATPase 2

A method for partial purification of ATPase 2 has previously been described [10]. The purification factor was only 134 times in comparison with the specific activity found in the mitochondrial fraction. This ATPase 2 was not homogeneous after polyacrylamide gel electrophoresis and we have tried to purify ATPase 2 by another purification procedure. The first two steps $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography) were performed as described previously [10]. After DEAE-cellulose chromatography, fractions containing ATPase 2 were precipitated with saturated $(NH_4)_2SO_4$ and dialyzed overnight against 1 mM phosphate buffer (pH 6.8).

Hydroxyapatite chromatography. The dialyzed solution was applied to a hydroxyapatite column previously equilibrated with 1 mM phosphate buffer (pH 6.8). After adsorption, the protein was eluted with a discontinuous gradient of 1 mM, 10 mM, 30 mM, 50 mM, 75 mM, 100 mM, 300 mM phosphate buffer (pH 6.8). ATPase 2 was eluted at a phosphate buffer concentration of 50 mM (Fig. 1). However, we have observed that an appreciable portion of ATPase activity was eluted with 30 mM phosphate buffer. Electrophoresis

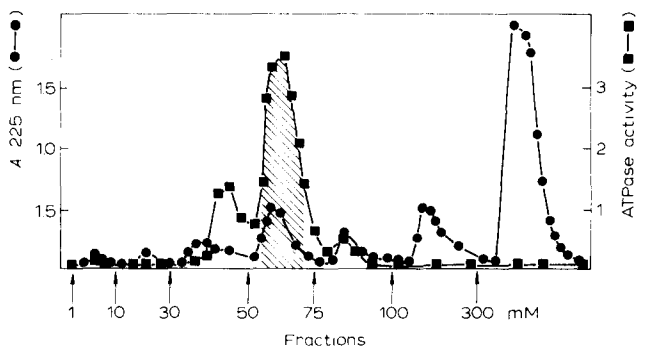

Fig. 1. Chromatography of ATPase on hydroxyapatite. 40—60 mg/20—30 ml of ATPase 2 fraction obtained by DEAE-cellulose chromatography were applied to a 2 × 8 cm column of hydroxyapatite. The flow rate was regulated by means of a peristaltic pump to approximately 30 ml/h. 4-ml fractions were collected. The absorbance at 225 nm (●———●) was measured and after having dialysed the fractions to eliminate phosphate buffer, the ATPase activity (■———■) of each fraction was determined by incubation of 50-μl aliquots in standard conditions. Successive phosphate buffer molarities are indicated by arrows. The active fractions pooled after chromatography are indicated by shading.

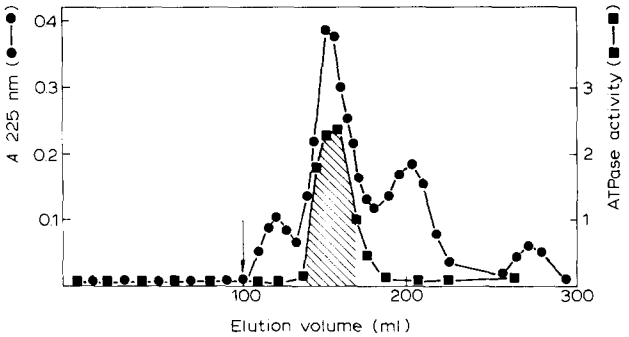

Fig. 2. Gel filtration on Sephadex G100. The solution (6—8 mg of the ATPase 2 fraction/1—2 ml) was applied to a column (2.6 ± 100 cm) of Sephadex G100. The eluent was 10 mM cacodylate/HCl buffer (pH 6.0)/0.1 m NaCl and the flow rate was 15—20 ml/h. The absorbance at 225 nm was measured (●———●) and ATPase activity (■———■) was determined by incubating 25-μl aliquots in the standard assay conditions. Exclusion volume of the gel is indicated by an arrow.

studies have shown that this enzyme was the same as that eluted at 50 mM. This fraction of the enzyme activity was rechromatographed on the column in a following preparation. This step eliminates cytochrome contaminations. Active fractions were collected and concentrated by precipitation with saturated $(NH_4)_2SO_4$.

Sephadex G100 filtration. The sample was applied to a Sephadex G100 column equilibrated with 10 mM cacodylate/HCl buffer (pH 6.0)/0.1 M NaCl (Fig. 2). ATPase 2 was eluted at 154 ml, the bed volume of the column being 114 ml. Enzymatic fractions, which corresponded to the shaded part of the figure, were pooled and (after $(NH_4)_2SO_4$ precipitation dialyzed overnight against 10 mM cacodylate/HCl buffer (pH 6.0).

AMP-Sepharose affinity chromatography. These fractions were not homogeneous after polyacrylamide gel electrophoresis and we tried to purify ATPase 2 by AMP-Sepharose affinity chromatography. Anderton et al. [20] showed that an (Na^+,K^+)-ATPase could be purified by ATP-Sepharose affinity chromatography. These authors have carried out an identical experiment on AMP-Sepharose column, but they indicated that the AMP analogue did not bind ATPase strongly and that AMP-Sepharose could also be used for the purification of other ATPases, which have a higher affinity for AMP than has the $(Na^+ + K^+)$-ATPase. For these reasons we chose to use an AMP-Sepharose column, since AMP is a competitive inhibitor and since, in our experimental conditions (20° C, pH 6.0), we thought that the ATPase would rapidly hydrolyze the ATP of an ATP-Sepharose column and that it would, therefore, be necessary to regenerate the column before use each time. It was possible to use an AMP-Sepharose column several times without measurable loss of binding capacity.

The experiment was carried out at 20° C, in order to obtain the best binding of the enzyme. 3 mg protein/2 ml 10 mM cacodylate/HCl (pH 5.8) was applied to an AMP-Sepharose column (Fig. 3). The ATPase 2 fraction was placed onto the column and it was washed with 10 mM cacodylate buffer until all the non-adsorbed proteins were eluted. A small part of the ATPase activity was asso-

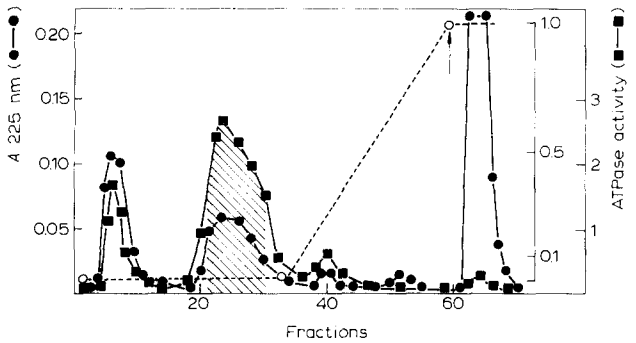

Fig. 3. Affinity chromatography of ATPase 2 on AMP-Sepharose column. 2 ml of partially purified ATPase 2 (1.5 mg/ml) obtained by Sephadex G100 filtration were applied to a column (0.5 × 3 cm) of AMP-Sepharose. At a flow rate of 10 ml/h, the peak containing high ATPase activity was retarded from the flow-through peak, and after an extensive washing (four bed volumes of 10 mM cacodylate/HCl buffer, pH 6.0). The column was developed with a KCl molarity gradient (0—1 m) 50 ml total volume in 10 mM cacodylate/HCl buffer, pH 6.0 (— — — —). The elution was terminated by applying a 500 μl pulse of 25 mM ATP (arrow). The column was run at 20°C and 2-ml fractions were collected. The absorbance at 225 nm (●————●) was measured and the ATPase activity (■————■) was determined by incubating 25-μl aliquots in the standard assay conditions. The fractions pooled after affinity chromatography are indicated by shading.

ciated with these non-adsorbed proteins. Most of the enzyme activity was retarded and could be subsequently eluted. Total retention of the enzyme was not observed but the simple retardation was sufficient to separate ATPase 2 from its contaminating proteins.

At this final step of the purification, the active fractions were collected (shaded zone Fig. 3) and concentrated. The yield of purified enzyme from 100 rat livers was approx. 1.5 mg. The purification factor in comparison with the specific activity found in the mitochondrial fraction was 242 times, which represents a purification factor of approx. 1500 in comparison to the original

TABLE I

PURIFICATION OF MITOCHONDRIAL SOLUBLE ATPase 2

Each experiment was performed with mitochondria from 100 rat livers. Results are given per rat liver and are the means of 10 experiments. The ATPase activities were determined by incubation of aliquot parts in standard deviations (means ± standard errors).

	Total activity (μmol $P_i \cdot min^{-1}$)	Protein (mg)	Specific activity (μmol $P_i \cdot min^{-1} \cdot mg^{-1}$ protein)	Purification	Yield (%)
Mitochondria	7.9 ± 1.2	150 ± 26	0.053	1	100
Mitochondrial soluble fraction	5.9 ± 0.6	48.3 ± 5.8	0.122	2.3	74.7
$(NH_4)_2SO_4$ precipitate	3.9 ± 0.4	12.9 ± 1.2	0.302	5.7	49.4
DEAE-cellulose	0.78 ± 0.08	0.52 ± 0.08	1.5	28.5	9.9
Hydroxyapatite	0.46 ± 0.07	0.070 ± 6.52	6.52	123	5.8
Sephadex G-100	0.28 ± 0.06	0.029 ± 0.009	9.8	131	3.5
AMP-Sepharose afffinity chromatography	0.21 ± 0.04	0.016 ± 0.005	12.8	242	2.7

homogenate (Table I). This moderate purification factor can be explained by the fact that the enzyme is partially inactivated during the purification (which takes 10—12 days). The purified fractions were precipitated with saturated $(NH_4)_2SO_4$ and stored in small amounts at −40° C in 10 mM cacodylate/HCl buffer (pH 6.0)/0.5 M $(NH_4)_2SO_4$ (to avoid inactivation which occurs when the enzyme is stored at 0° C or submitted to several freezings and thawings).

Criteria of purity

In order to assess the purity of our preparation we have performed electrophoresis on polyacrylamide gels under different conditions.

Non-denaturing electrophoresis on polyacrylamide gels. Following electrophoresis under these conditions, we have observed a single protein band (Fig. 4a). We have also measured the enzymatic activity from gel slices. Fig. 4a shows an exact correlation between the densitometric trace at 540 nm and the enzymatic activity which proves that the ATPase 2 was localized within the protein band.

We have also used bidimensional gel electrophoresis and this technique allowed a more exact indication of purity, because the proteins were separated by function of their charge in the first dimension and by function of their molecular weight in the second. Only one spot was observed on the slab after migration in the second dimension (Fig. 4b). By dividing the slab in half, we

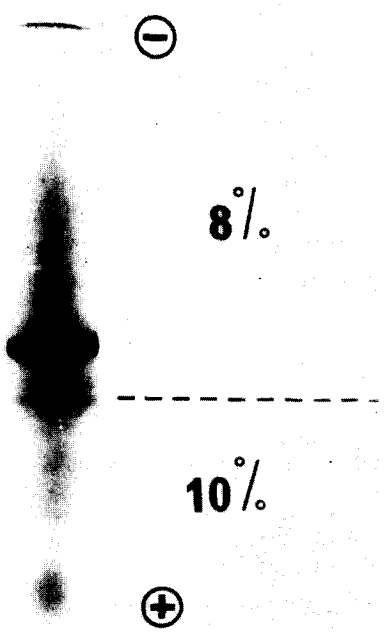

Fig. 4. Electrophoresis of ATPase 2 in double polyacrylamide gel system in the presence of SDS. 30 μg protein were applied to the top of the polyacrylamide gel (8% upper part and 10% lower part). The gels were polymerized in the presence of 0.1% SDS. The electrophoresis running buffer was Tris/glycine (pH 8.3). The run was performed at room temperature at 1 mA/tube and electrophoresis was continued for approx. 3 h. The gels were stained in Coomassie brillant blue. The stippling indicates the gel separation.

showed that the enzymatic activity was localized on the same level as the protein spot.

Electrophoresis in denaturing conditions in the presence of SDS. After electrophoresis in a double polyacrylamide gel system (8—10%) in the presence of 0.1% SDS, only one band of protein was observed when 10—50 μg ATPase 2 were applied. Only one band was also seen in the presence of 0.1% SDS, 6 M urea and 1 mM thioglycolic acid.

Molecular weight determination. The molecular weight of ATPase 2 was determined by Sephadex G100 filtration in the presence of several markers. The molecular weight measured by this method was found to be 61 500 ± 3000. When acrylamide gel electrophoresis in presence of SDS, urea and thioglycolic acid was used for molecular weight determination, the molecular weight of ATPase 2 was also found to be 61 500 ± 3000.

Discussion

We have shown previously that the ATPase activity isolated from the soluble fraction of rat liver mitochondria is associated with distinct molecular forms [10,11]. It was possible to resolve these two forms by DEAE-cellulose chromatography. We have purified ATPase 2 about 1500 times in comparison to the original rat liver homogenate and the efficiency of this purification was mainly due to the use of an AMP-Sepharose affinity column. We examined the purity of our preparation by several criteria. This study has shown that the two forms behave differently during the purification procedure. ATPase 1 was not retained by DEAE-cellulose chromatography, while ATPase 2 was retained. On the other hand, using AMP-Sepharose affinity chromatography, ATPase 1 was adsorbed on the column and ATPase 2 was only retarded. The two ATPases have been shown to have separate physico-chemical properties and molecular weight and are, thus, distinct enzymatic forms sharing the property to hydrolyze nucleoside triphosphates. ATPase 2 differs also from F1 fraction, since its molecular weight is different from the molecular weight of all its subunits. We are interested in the metabolic role of these enzymes in the liver mitochondria and we are also examining brain mitochondria for the presence of these enzymes.

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