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Affinity chromatography of mitochondrial ATPase dispersed with Triton X-100

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SUMMARY

Mitochondrial ATPase, dispersed with Triton X-100, can be bound to a column of mitochondrial ATPase inhibitor protein covalently coupled to Sepharose 4B. The extent of binding is dependent on the presence of Mg^{2+} and ATP. The bound ATPase is detached by increasing the pH and the ionic strength and has a specific activity of up to 9 times that of the starting material.

Affinity chromatography, based on biospecific adsorption between molecules, has so far found little use in the purification and study of membrane-bound enzymes with the notable exception of glycoproteins. The mitochondrial ATPase appears to offer a unique opportunity to utilize this technique, due to the existence of a small soluble protein which is a specific inhibitor of this enzyme[1]. In this report we wish to show that ATPase inhibitor which is covalently coupled to an agarose matrix, can indeed bind ATPase in a Triton extract and that this interaction can be used to purify ATPase from submitochondrial particles.

ATPase inhibitor was prepared as described by Horstman and Racker[2]. 0.5 mg of the purified inhibitor was coupled to 0.5 g CNBr-activated Sepharose 4B (trademark product of Pharmacia Fine Chemicals, Uppsala, Sweden), following the manufacturer's instructions. The inhibitor–Sepharose gel was packed into a column of 0.95-cm length and 1.40-cm diameter. Before each use the column was equilibrated with Solution A, containing 0.2 M sucrose, 15 mM Tris–*N*-tris(hydroxymethyl)methyl-2-aminoethane

Abbreviation: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid.

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sulphonic acid (TES) buffer (pH 6.6), 3 mM KCl, 0.5 mM $MgSO_4$, 0.5 mM ATP, 5 mM phosphoenolpyruvate, 60 μg/ml pyruvate kinase (Boehringer, Mannheim, Germany) and 3 mg/ml Triton X-100 (purchased through Sigma Chemical Co., St. Louis, Missouri, U.S.A.). After use the column was washed with 10 ml acetate (pH 4), 1 M NaCl, followed by 10 ml borate (pH 8), 1 M NaCl, and was then ready for re-equilibration with Solution A. For longer periods between usage the column was stored at 4 °C in the presence of penicillin and streptomycin.

Tris–TES buffers were prepared by mixing equimolar solutions of Tris and TES to give the desired pH. The concentration of the buffer is given as the combined concentrations of Tris and TES.

ATPase was determined enzymically[3,4]. Protein was measured using the method described by Bramhall *et al.*[5], but replacing xylene brilliant cyanine with amido black.

Submitochondrial particles (EDTA particles) were prepared as described by Lee and Ernster[6]. 120 mg of particles were sedimented at 104 000 × *g*, 30 min, 0 °C, and were resuspended in 6 ml 0.2 M sucrose, 15 mM Tris–TES buffer (pH 6.6) and 3 mg/ml Triton X-100. The suspension was centrifuged as above and the yellowish supernatant was collected. This supernatant, termed the Triton extract, contained 0.75 mg/ml protein and an ATPase activity of 2.1 μmoles/min per mg protein. To 4.5 ml Triton extract we added KCl, $MgSO_4$, phosphoenolpyruvate, pyruvate kinase and ATP, in that order, to reach the final concentrations of Solution A. The extract was then applied to the column, followed by 1 ml Solution A. Then 5 ml of Solution B, containing 0.2 M sucrose, 15 mM Tris–TES buffer (pH 6.6), 0.5 mM $MgSO_4$, 0.5 mM ATP and 0.3 mg/ml Triton X-100 were passed through the column. Finally, the column was eluted with Solution C, containing 0.2 M sucrose, 45 mM Tris–TES buffer (pH 8.75), 0.5 M KCl, 1 mM EDTA and 0.3 mg/ml Triton X-100. The column was operated at room temperature, using a flow rate of 6.30 ml/h.

The elution pattern is shown in Fig. 1. Most of the Triton extract passes directly through the column, without purification, and is eluted in the first peak. However, some ATPase appears to be attached to the column and is eluted in a second peak only after Solution C is applied to the column. The second peak shows a several-fold increase in specific activity over the Triton extract. This increase is directly proportional to a decrease in absorbance at 415 nm. The maximal specific activity is trailing slightly behind the peak of total activity.

A number of factors influence the binding of ATPase to the inhibitor–Sepharose gel. A pH below 7, a low ionic strength and the presence of ATP and Mg^{2+} have been shown to be important for the efficiency of ATPase inhibition by the inhibitor[1,2]. Thus a low pH and a low ionic strength are used to attach the ATPase, whereas the bound ATPase can be eluted from the column by increasing the pH and the ionic strength. We also found that the yield of ATPase attached to the column is dependent on the presence of Mg^{2+} and ATP, as shown in Table I. Since ATP added to the Triton extract rapidly will be hydrolyzed by the ATPase and a high ATP/ADP ratio has been reported to enhance the efficiency of the inhibitor[7], we decided to have an ATP-regenerating system present during binding.

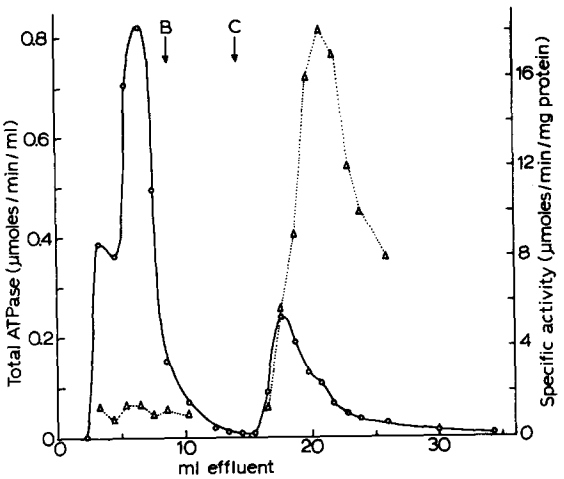

Fig. 1. Affinity chromatography of mitochondrial ATPase, dispersed with Triton X-100, on a column of ATPase inhibitor covalently coupled to Sepharose 4B. Experimental details are given in the text. o—o, the total ATPase activity; Δ . . . Δ, the specific activity. Arrows B and C mark the start of elution with Solution B and C, respectively.

TABLE I

EFFECT OF Mg^{2+} AND ATP ON THE AMOUNT OF ATPase BOUND TO AN ATPase INHIBITOR–SEPHAROSE COLUMN

Experimental conditions essentially as in the text, except that Solution A was replaced with 0.2 M sucrose, 15 mM Tris–TES (pH 6.6), 3 mg/ml Triton X-100 and additions as indicated in the table. The amount of enzyme bound is calculated as the amount eluted with Solution C.

Additions	*ATPase applied (nmoles/min)*	*ATPase bound (nmoles/min)*	*Yield (% bound of applied)*
None	336	39	12
1 mM $MgSO_4$	192	35	18
0.5 mM $MgSO_4$ + 0.5 mM ATP	678	221	33

The yield of ATPase in the second peak, Fig. 1, is about 13% of the total ATPase in the Triton extract. As is evident from Table II, the yield is increased by using small sample loads but the total amount of ATPase attached is also much smaller. Only with excessive sample loads is the maximum capacity of the column used, which indicates that the equilibrium of the reaction towards the formation of an ATPase–ATPase inhibitor complex is rather unfavourable. A slower flow rate in the column gives no appreciable increase in yield. On the other hand, there is virtually no detachment of enzyme once it is bound. One explanation for this behaviour would be that the ATPase interacts with the inhibitor only in a certain state, the formation of which is promoted by the presence of ATP and Mg^{2+}.

TABLE II

EFFECT OF SAMPLE LOAD ON THE AMOUNT OF ATPase BOUND TO AN ATPase INHIBITOR–SEPHAROSE COLUMN

Experimental conditions are given in the text. The amount of enzyme bound is calculated as the amount eluted with Solution C.

ATPase applied (nmoles/min)	*ATPase bound (nmoles/min)*	*Yield (% bound of applied)*
424	146	34
7110	944	13

The ATPase eluted in the second peak is cold stable and sensitive to oligomycin, albeit only at very high concentrations of the antibiotic (10 μg/ml gives 50% inhibition with 3 μg/ml ATPase and 30 μg/ml Triton present). These properties distinguish our ATPase activity from that of coupling factor 1 (ref. 3). The enzyme may be similar to the Triton-dispersed ATPase from yeast mitochondria, described by Tzagoloff and Meagher[8]. Thus the high concentrations of oligomycin required for inhibition may reflect a competition between Triton and oligomycin, along the lines suggested by these authors. Gel chromatography of beef heart ATPase in the presence of Triton indicates that the enzyme is small enough to penetrate into Sepharose 6B gel.

On the basis of the results reported here, we feel that affinity chromatography can be used as a specific, simple and, once the inhibitor–Sepharose gel is prepared, rapid purification step for mitochondrial ATPase dispersed with detergent. The possibility to study the interaction between ATPase and the ATPase inhibitor protein, when the latter is bound to a solid support, should also provide a valuable tool in the evaluation of the mechanism of inhibition.

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