ATP-ANALOGUE-BOUND GEL MATRIX AND ITS USE AS AN AFFINITY ADSORBENT FOR Na^+, K^+-ATPase

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1. Introduction

The use of nucleotides and cofactors as bound ligands in affinity chromatography has been the subject of a number of recent papers [1–5]. There have been two reports of the synthesis of matrix-bound cyclic-AMP [3, 4]. Tesser et al. [3] reported a degree of instability in their bound cyclic AMP derivative. During the preparation of this manuscript a procedure was published describing the synthesis of an ATP–agarose affinity chromatography material, this material has not yet been used for the separation of ATP liganding proteins [5].

We wish now to report the synthesis of an ATP-analogue-to-agarose affinity column. Such a column binds detergent solubilized Na^+, K^+-ATPase (EC 3.6.1.3), and the bound enzyme can be eluted with an ATP solution. The column has the potential to be a very useful tool for purification of some of the many enzymes, which possess ATP-binding sites. Among such enzymes are several membrane-bound proteins and affinity chromatography promises to be a useful method for the purification of this group of proteins.

2. Materials and methods

The starting material for the ATP analogue synthesis, 6-mercaptopurine riboside was obtained from Waldhof Chemie, Mannheim. Diaminopropylamine and *N*-acetylhomocysteine thiolactone were obtained from R.N.Emmanuel, Ltd., Wembley, Middlesex.

$ATPNa_2$, phosphoenolpyruvate-tricyclohexylammonium salt, pig muscle lactate dehydrogenase (EC 1.1.1.27), NADH and rabbit muscle pyruvate kinase (EC 2.7.1.40) were purchased from Boehringer Mannheim, London. Ouabain, Brij 58 and 'Trizma' base were from Sigma, London.

6-(Purine 5'-ribosyltriphosphate)-4-(1,3-dinitrophenyl) thioether (ATP-analogue) was synthesized from the isopropylidated riboside. The nucleoside was first converted to the thioether by reaction with fluoro-dinitrobenzene, then phosphorylated with the aid of $POCl_3$. The monophosphate was reacted with diphenylchlorophosphate, and the resulting compound yielded the triphosphate upon addition of pyrophosphate. The details of the synthesis will be published elsewhere [6].

The thioether bond of the ATP derivative reacts readily with aliphatic thiol compounds to form very stable thioethers [7]. The ATP-analogue was coupled to thiolated Sepharose 4B (Pharmacia) in 0.1 M Tris–chloride buffer at pH 8 during a 1 hr incubation. The ATP-analogue–Sepharose was then used to pour a column and the column equilibrated with the appropriate buffer for chromatography of the ATPase. After use, the ATP–Sepharose was washed with distilled water and stored frozen (–16°C).

Thiolated Sepharose 4B was prepared from Sepharose pretreated with epichlorohydrin and sodium borohydride [8] prior to activation with cyanogen bromide, amination with 3,3′-iminobispropylamin, and final coupling with *N*-acetyl-homocysteine thiolactone at pH 9.7 [9]. This produced Sepharose with 10 μmoles thiol groups per gram of packed wet weight as determined by reaction with dithionitrobenzoate (DTNB). Thiolated Sepharose was stored in 5 mM dithiothreitol under nitrogen at 5°C.

ATPase activity was measured at 37°C in approximately 100 mM Tris buffer pH 7.4 by coupling the production of ADP to NADH consumption with pyruvate kinase (PK) and lactate dehydrogenase (LDH). Initial rate determinations of the hydrolysis of ATP could then be followed by the decrease in absoption at 340 nm in a Pye-Unicam SP 800 recording spectrophotometer. The assay volume was 1.00 ml containing 2 mM ATP, 1.5 mM phosphoenolpyruvate, 0.23 mM NADH, 3 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 4.5 units of LDH, and 3.75 units of PK activity. This gave approximately 6.5 mM $(NH_4)_2SO_4$ from the stock $(NH_4)_2SO_4$ suspensions of PK and LDH. All assays were allowed to proceed until a sufficiently long linear trace was obtained and then 25 μl of ouabain were added to give a final ouabain concentration of 0.1 mM, and the assay was continued. Ouabain-sensitive (Na^+, K^+) ATPase was then taken as the difference between the slopes of the two traces, the dilution being virtually insignificant.

(Na^+, K^+) ATPase was prepared from bovine brain tissue. Cortex from 8 to 12 cattle brains was removed and homogenized cold in a Waring blendor as a 20% (w/v) homogenate in 0.32 M sucrose, 1 mM EDTA at pH 7.0. The homogenate was centrifuged for 15 min at 10 000 *g* in the 6 × 250 ml rotor of the MSE Angle 18 centrifuge. The supernatant was retained and the pellets resuspended in their original volume of sucrose solution and recentrifuged for 15 min at 10 000 *g* and the supernatant combined with the first. The total supernatant was then centrifuged for 3 hr at 22 000 *g* and the resultant pellets resuspended to a final protein concentration of about 60 mg/ml in 1 mM EDTA at pH 7.0. This microsomal fraction was divided into 5 ml aliquots and stored at –16°C.

ATPase was solubilized from the microsomal material with the detergent Brij 58. The detergent was dissolved in 1 mM EDTA, pH 7.0, and then passed through a 0.45 μ filter (Millipore Corp.). Microsomal suspensions (adjusted to pH 7.0; 60 mg/ml) were mixed with an equal volume of 10% (w/v) Brij 58/EDTA and allowed to stand at 0°C for 15 min. The mixture was centrifuged for 45 min at 50 000 rev/min in a type 65 rotor on a Beckman L2-65B centrifuge. The supernatant contained, among other proteins, some solubilized ATPase (approximately 15% of the total ATPase activity of the original microsomal preparation).

3. Results and discussion

The reaction of the ATP analogue with the thiolated Sepharose can be followed by the release of the coloured dinitrophenyl mercaptide; the expected second product of the reaction for the production of a thioether bond between the ATP analogue and the thiolated Sepharose.

In a typical preparation 10 mg of the ATP analogue were reacted with 12 ml (packed volume) of thiolated Sepharose. This amount of ATP analogue was not sufficient to react with all of the available thiol groups. Fig. 1 illustrates the reaction of the ATP analogue with the thiolated Sepharose and the structure of the affinity column material so produced.

A column of bed volume 12 ml (10 cm × 1.25 cm) was equilibrated with 20 mM imidazole buffer, 1 mM EDTA, 0.01% Brij 58 at pH 6.5 ('running buffer'). One ml of the solubilized ATPase preparation containing 13 units (1 unit of ATPase activity liberates 1 μmole of phosphate per hour at 37°C) of (Na^+, K^+) ATPase activity was loaded onto the column and the column then washed with two bed volumes of running buffer at a flow rate of 6 ml/hr. The column was run at 7°C and 2 ml fractions were collected. The column was then washed with 2.5 mg/ml solution of

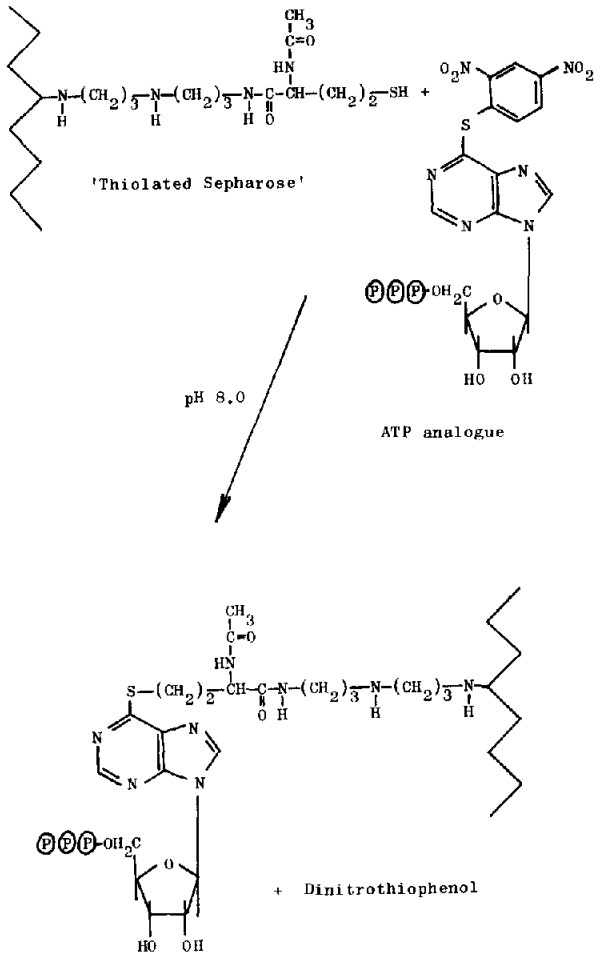

Fig. 1. Reaction scheme for the ATP analogue with thiolated Sepharose and probable structure of affinity matrix used for the chromatography of the solubilized Na^+, K^+-ATPase.

ATP in the running buffer. Fig. 2 shows the elution profile from such an experiment. There was a small peak of essentially ouabain-insensitive ATPase activity in the void volume. A sharp peak of mostly ouabain-sensitive ATPase activity emerged with the front of the ATP solution; there was a noticeable tailing of this peak. The total recovery of (Na^+,K^+) ATPase activity from the column was 4.5 units, which represents 35% recovery of the activity added to the column. This rather low recovery results from the known instability of this enzyme, once solubilized [10].

We have performed similar experiments at pH 6.0 and pH 7.6. The stability of the ATPase is greater at lower pH, but the binding to the column is much stronger at higher pH values; pH 6.5 was used as a reasonable compromise. The binding at these pH values of the Na^+,K^+-ATPase to the column material is unlikely to be simply a non-specific ionic interaction as this enzyme behaves as an anion above pH 5.5 [11].

Column material has been used several times without noticeable loss of binding capacity.

We have also prepared Sepharose with similarly bound AMP from the equivalent AMP analogue [7]. This material does not bind ATPase strongly, which indicates that the binding observed with the ATP-analogue–Sepharose is a property of the triphosphorylated ligand. AMP–Sepharose may be of use for purification of other enzymes, which have a higher affinity for AMP than has the Na^+,K^+-ATPase.

Work is now in progress on the further purification of the Na^+,K^+-ATPase and application to other ATP binding enzymes using this method. It may also be possible to convert the bound ATP to cyclic AMP.

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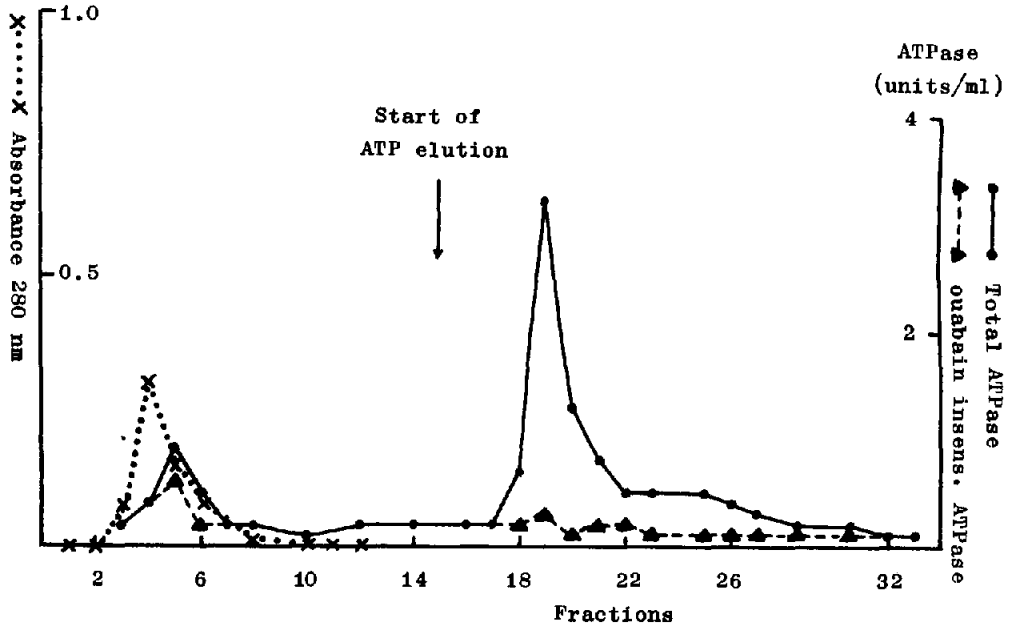

Fig. 2. Elution profile of solubilized ATPase charged column. 13 units of solubilized ATPase were loaded onto the column and 2 bed volumes of buffer allowed to pass through. After fraction 15 had been collected the column was eluted with a 2.5 mg/ml solution of ATP in running buffer. For further details see the text. (x...x...x, absorbance at 280 nm; ●—●—●, total ATPase; ▲– – –▲– – –▲ ouabain insensitive ATPase). The difference between total ATPase and ouabain-insensitive ATPase represents the Na^+,K^+-ATPase.

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