A facile method for the preparation of N⁶-substituted ATP-Sepharose

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ABSTRACT

The attachment of 6-methylsulfonylpurineribose 5'-triphosphate to AH-Sepharose 4B as a facile method for the preparation of ATP-Sepharose is described. This reaction is compared to the coupling of 6-chloropurineriboside 5'-triphosphate as well as N⁶-aminocaproyl-ATP. An improved synthesis of purineriboside 5'-phosphates which does not require purification by column chromatography is also described.

INTRODUCTION

The attachment of AMP- and NAD-derivatives to Sepharose has been reported in many cases for the purpose of affinity chromatography (1,2). Many of the procedures developed for these compounds cannot be necessarily applied to the attachment of ATP to Sepharose. However, derivatives of ATP have been coupled to Sepharose and other polymers (3) by the terminal phosphate (3,4), the ribose (5), the C-8 position (3,6) and the N⁶-position (3,6,7,8). The procedures for the latter are rather time consuming. We were particularly interested in finding a facile reaction to attach ATP to Sepharose through the N⁶-position in order to use the ATP-Sepharose for the isolation of ATPases. We intended to make use of the nucleophilicity of the amino group of the aminohexyl side arm of the commercially available AH-Sepharose 4B. As potential leaving group in the 6-position of the nucleoside 5'-triphosphate we envisaged the recently described (9) methylsulfonyl group which at least in simple nucleophilic substitution reactions in homogeneous solution is considerably more reactive than the chloro-group. We therefore set out to compare the efficiency of attachment of several ATP derivatives to Sepharose (Scheme I).

The results indicate that coupling 6-chloropurineriboside 5'-triphosphate to AH-Sepharose 4B does not result in a good yield of attachment.

RTP: RIBOSE 5-TRIPHOSPHATE

SCHEME I

However, the 6-methylsulfonyl derivative is at least 15 times more reactive and is comparable in yield to the coupling of aminocaproyl-ATP to activated Sepharose. Considering the availability of the substituted Sepharose and the ease of preparation of the 6-methylsulfonyl compound - the oxidation and purification is carried out in about 30 min - this coupling procedure seems to represent an easy method. If a higher concentration of ligand is desired, Sepharose with a higher amine content can easily be prepared by known procedures.

In the course of this study we observed that the phosphorylation of purine nucleosides by POCl_3 in triethylphosphate (10) can be carried out in such a way that the isolation of the nucleoside monophosphate can be achieved without purification by column chromatography, thus greatly facilitating this reaction.

MATERIALS AND METHODS

6-Chloropurine riboside and 6-methylmercaptopyrimidine riboside were products of Papierwerke Waldhof-Aschaffenburg, Mannheim, Germany. 2',3',5'-

Triacetyladenosine was prepared according to the literature (11). AH-Sepharose 4B was obtained from Pharmacia. Alkaline phosphatase (calf intestine, 1 mg/ml, 400 U/mg) was purchased from Boehringer, Germany. The nucleoside 5'-triphosphates were synthesized from the corresponding monophosphates according to Michelson (12) and analyzed by electrophoresis at pH 3.5 (0.05 M ammonium formate). Sepharose was activated with CNBr as described (13). Phosphate was determined according to Chen et al. (14); to about 1 ml of substituted gel was added 500 μ l 0.1 M Tris pH 8.9 and 100 μ l alkaline phosphatase and after one hour stirring at 37°C it was centrifuged and 200 μ l of the supernatant used for the phosphate determination.

N⁶-Benzyloxycarbonylaminoacroyl-adenosine: 2',3',5'-Triacetyladenosine (1.65 g, 5 μ moles) was reacted with N-carbobenzoxycarboxylanhydride (15) (9 g, 18 μ moles) in pyridine (12 ml) for 5 hrs at 80°C, the solvent completely evaporated, the residue dissolved in ethanol (40 ml), 2 N NaOH (20 ml) added and the solution neutralized after 2.5 min with acetic acid (approx. 1.5 ml). After evaporation to dryness, the residue was suspended in H₂O (approx. 100 ml), filtered by suction and the material recrystallized from methanol. Yield 1.0 g (39 %), mp 163-165°C. Anal. calcd. for C₂₉H₃₀N₆O₇: C 56.02; H 5.87; N 16.33. Found: C 56.34; H 6.04; N 16.48.

Phosphorylations: Nucleosides (1.0 μ mol) were dried at 110°C in vacuo overnight and dissolved in triethylphosphate (2.50 ml), if necessary by gentle heating over an open flame. After cooling in ice, POCl₃ (110 μ l, 1.2 μ mol) was added, the solution left at +4°C for 2.5 hrs, aqueous barium acetate solution (10 %, 10 ml) added and the solution transferred to a centrifuge tube. Triethylamine (1.0 ml) was added, the precipitation completed by addition of 95 % aqueous ethanol (30 ml) and centrifuged after standing at +4°C for 10 min. The supernatant was decanted, the pellet washed with 70 % aqueous ethanol (3 x 20 ml) and then extracted with water (6 x 30 ml) until no more UV-active material could be dissolved. The combined aqueous solutions were evaporated to dryness and the residue analyzed by electrophoresis at pH 7.5 as well as by elemental analysis. These nucleoside 5'-phosphates were completely degraded by 5'-nucleotidase as judged by paper electrophoresis at pH 7.5.

The following nucleosides were phosphorylated in this manner:

- 1) N⁶-Benzyloxycarbonylaminoacproyladenosine (yield 560 mg, 77 %). Anal. calcd. for C₂₄H₂₉N₆O₁₀PBa (729.87): N 11.51; P 4.24. Found: N 11.46; P 4.75.
- 2) 6-Chloropurine riboside (yield 225 mg, 56 %). Anal. calcd. for C₁₀H₁₀ClN₄O₇PBa (502.00): Cl 7.06; N 11.16; P 6.16. Found: Cl 6.52; N 10.34; P 5.80.
- 3) 6-Methylmercaptapurine riboside (yield 265 mg, 52 %), $\lambda_{\max}^{\text{H}_2\text{O}}$ 290 nm (16 300). Anal. calcd. for C₁₁H₁₃N₄O₇SPBa (513.64): C 25.72; N 10.90; P 6.03. Found: C 25.48; N 10.83; P 6.10.

6-Methylsulfonylpurineriboside 5'-triphosphate: Chlorine gas was bubbled through an aqueous solution (2 ml) of 6-methylmercaptapurine riboside 5'-triphosphate (28 μ moles), cooled in ice (9). The progress of oxidation was followed by taking UV-spectra at short intervals. The reaction is associated with a shift of absorption from 290 nm to 280 nm and a large drop in extinction coefficient. As soon as the reaction was completed (about 5-15 min) the flow of chlorine was stopped, N₂ bubbled through for a few minutes at 0°C and the solution neutralized with 1 M NaHCO₃ solution. The volume was reduced to about 0.5 ml at 25°C first with a water aspirator to degas the solution, then with an oil pump. The pH of this solution was adjusted to about 5 and remaining chlorine removed by passing over a small Sephadex G-10 column which was eluted with water. The triphosphate was eluted in the first fractions which were then reduced in volume to the desired concentration by evaporation at 25°C. The yield is 85 % ($\lambda_{\max}^{\text{H}_2\text{O}}$ 278 nm (8 700)).

N⁶-Aminocaproyl-adenosine 5'-triphosphate: N⁶-Benzyloxycarbonylamino-caproyl-adenosine 5'-triphosphate (120 μ moles) and 75 μ l triethylamine were dissolved in 20 ml 50 % aqueous methanol and hydrogenated in the presence of Pd/C (10 % Pd) for 1 hour under slightly elevated pressure. After filtration the material was chromatographed on a DEAE-Sephadex column with a linear gradient of 500 ml each of 0.1 M and 0.6 M triethylammoniumbicarbonate pH 7.5. Yield 40 % ($\lambda_{\max}^{\text{H}_2\text{O}}$ 272 nm (14 500)).

For the coupling of 6-methylsulfonylpurineriboside 5'-triphosphate to AH-Sepharose 4B, 2.5 g of the washed Sepharose was added to a solution (6 ml) which was 2.5 mM in triphosphate and 0.1 M in Hepes pH 8.0. After gentle stirring for 2 hours at room temperature the gel (about 10 ml) was put in a column and thoroughly washed with 0.1 M imidazole pH 7.0 (total

volume 400 ml). From the UV-absorption at 280 nm it was estimated that 0.23 μ moles triphosphate were bound/ml of gel. Phosphate analysis indicated that about 0.15 μ mol of triphosphate were bound/ml of gel. 6-Chloro-purineriboside 5'-triphosphate was coupled by the same procedure but the reaction time extended to 12 hours. Only about 10 nmol triphosphate were bound/ml gel in this reaction as judged from the phosphate analysis. As a comparison for the alternative reaction, nucleophilic attack of the triphosphate on Sepharose, N⁶-aminocaproyl-ATP (65 μ moles) was coupled in 0.1 M NaHCO₃ pH 9.0 (25 ml) to CNBr-activated Sepharose (25 ml) at 4°C for 12 hours. Phosphate analysis indicated 0.25 μ moles ATP/ml gel.

For all three preparations phosphate analyses by combustion with Mg(NO₃)₂ yielded values which were very similar to those obtained by phosphatase degradation.

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