PURIFICATION OF *ESCHERICHIA COLI* ADPGLUCOSE PYROPHOSPHORYLASE BY AFFINITY CHROMATOGRAPHY*

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1. Introduction

Purification schemes using conventional techniques have been published for a number of ADPglucose pyrophosphorylases [1–3]. However, they have resulted either in low yields of the enzyme or in tedious and lengthy procedures. The synthesis of a Sepharose derivative with a ligand resembling fru-P_2, the allosteric activator of the *Escherichia coli* ADPglucose pyrophosphorylase [4], and its effectiveness in facilitating the purification of an ADPglucose pyrophosphorylase from an *E. coli* B mutant, AC70R1, derepressed in the synthesis of the glycogen biosynthetic enzymes are reported below.

2. Materials and methods

2.1. *Assay of ADPglucose pyrophosphorylase*

Formation of [^{32}P] ATP from ADPglucose and $^{32}PP_i$ was assayed as previously described [5]. The reaction mixture contained 20 μmoles of Tris–Cl buffer (pH = 8.0), 100 μg of bovine plasma albumin, 2.0 μmoles of $MgCl_2$, 0.2 μmole of ADPglucose, 0.5 μmole of $^{32}PP_i$ (5×10^6 to 30×10^6 cpm per μmole), 0.4 μmole of fru-P_2, 2.5 μmoles of NaF, and enzyme in a volume of 0.25 ml. A unit of enzyme is that amount that catalyzes the conversion of one μmole of ADPglucose to ATP in 1 min under the above conditions at 37°C.

2.2. *Analytical procedures*

Descending paper chromatography was carried out on Whatman No. 1 with 95% ethanol–1 M aqueous ammonium acetate buffer pH 3.6 (5:2, v/v) as solvent 1 and concentrated ammonium hydroxide–methanol–water (2:7:1, v/v) as solvent 2. Chromatograms were developed with ninhydrin for amines [6]: $FeCl_3$ and sulfosalicylic acid for phosphates [7].

Electrophoresis was performed on 10% polyacrylamide gels in sodium dodecyl sulfate [8] and on analytical disc gel electrophoresis using the Tris–glycine buffer system [9]. Protein concentrations were estimated by the method of Lowry et al. [10]. Phosphate concentrations were estimated as described by Fiske and Subbarow [11].

Reagent grade solvents were used without further purification and were dried over 4 Å molecular sieves (Fischer). 1,6-Hexanediol diphosphate [12], *N*-trifluoroacetyl 6-amino-1-hexanol phosphate imidazolide [13] (Compound I; fig. 1), and methyl tri-*n*-octylammonium hydroxide [14] were synthesized as described.

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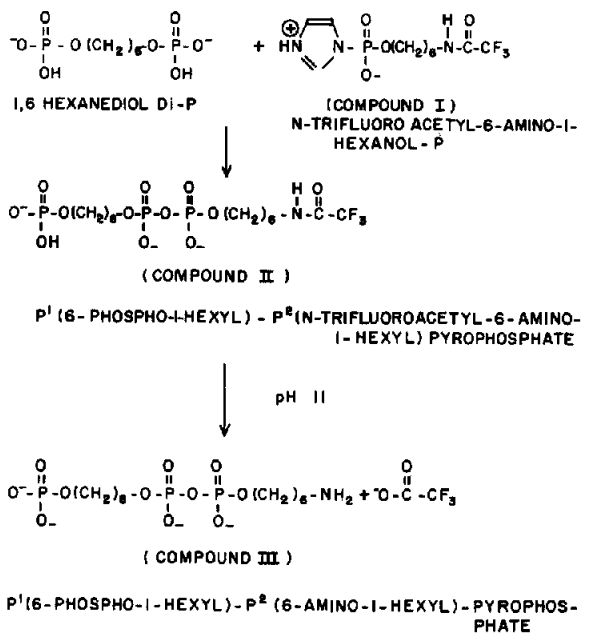

Fig. 1. Synthesis of P^1-(6-phospho-1-hexyl)-P^2-(6-amino-1-hexyl)-pyrophosphate (Compound III).

2.3. *Synthesis of P^1 (6-phospho-1-hexyl)-P^2-(6-amino-1-hexyl) pyrophosphate*

(Compound III; fig. 1). Hexanediol diP (acid form), 2.5 mmoles, was mixed with 5.0 mmoles methyl-tri-*n*-octylamine and methyl alcohol, 50 ml, was added to give a homogeneous solution. The solvent was removed in vacuo, dried by addition and evaporation of anhydrous dimethyl formamide and stored in vacuo over drierite. *N*-Trifluoro-6-amino-1-hexanol phosphate imidazolide, 2.55 mmoles, was dissolved in 5 ml of dimethyl formamide and added to 2.5 mmoles of the hexanediol-diP-methyl-tri-*n*-octylamine salt dissolved in 6 ml dimethyl formamide. This formed a homogeneous solution which was stored in a dessicator for 24 hr. This mixture was then absorbed onto a 2.5 cm × 30 cm column of Dowex 1 × 8 – 200–400 mesh (Cl^- form), washed with 300 ml of 50% methanol, and then eluted with a linear gradient consisting of 2 liters of 0.01 M HCl in the mixing chamber and 2 liters of 0.01 M HCl and 0.6 M LiCl ir the reservoir. Fractions were analyzed for total phosphate to locate products of the reaction. The tubes containing P^1 (6-phospho-1-hexyl)-P^2 (*N*-trifluoroacetyl-6-amino-1-hexyl) pyrophosphate (Compound II; fig. I) were pooled, adjusted to neutrality with 2.5 M LiOH and concentrated to 75 ml. This yielded a solution which contained 2.5 mmoles of total phosphate. The solution was adjusted to pH 11 with 2.5 M LiOH and incubated at room temperature for 4 hr, and then neutralized with HCl. Incubation at pH 11 results in the release of trifluoro-acetate from Compound II. The residue left after flash evaporation containing Compound III was extracted several times with ethanol–diethylether (1:2, v/v) to remove LiCl. Chromatography of the Compound III in solvent system 1 and 2 gave a single ninhydrin positive spot which contained phosphate. Hydrolysis for 30 min in 1 N HCl at 100°C gave two spots containing phosphate. One of these was ninhydrin positive and cochromatographed with 6-amino-1-hexanol phosphate and the other cochromatographed with 1,6 hexane-diol diphosphate and was ninhydrin negative. The stoichiometry of ninhydrin reacting material (using hexanolamine-P as a standard) to total phosphate in compound III was 1.0 to 3.3. Negligible amounts of acid-labile or inorganic phosphate were found.

2.4. *Coupling of Compound III to Sepharose 4B*

Compound III was coupled to Sepharose 4B by the cyanogen bromide technique [15]. About 600 μmoles of the ligand were used to react with 75 ml packed resin. Phosphate analysis indicated that 50% of the ligand had reacted giving a resin with 2.8 μmoles of ligand per ml of packed resin.

2.5. *Preparation of ADPglucose pyrophosphorylase*

The enzyme was purified from a mutant strain of *E. coli* B, AC70R1. The strain was isolated from cells mutated with nitrosoguanidine [16]. Putative glycogen mutants were visualized with iodine staining as previously described [17]. AC70R1 stains very much darker than the parent strain. It contains 6- to 10-times higher levels of ADPglucose pyrophosphorylase than *E. coli* B when expressed on a unit/mg-protein basis. The kinetic constants for substrates, activators and inhibitors of the AC70R1 ADPglucose pyrophosphorylase were found to be similar to those found for the *E. coli* B enzyme [18]. Cells were grown and proteins purified through the DEAE-cellulose chromatography step essentially as previously described [3]. The partially purified enzyme obtained from this proce-

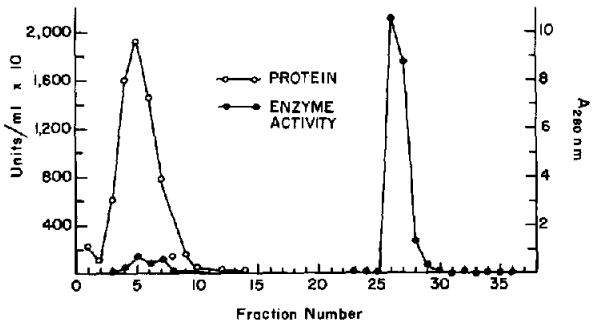

Fig. 2. Chromatography of ADPglucose pyrophosphorylase on Compound III–Sepharose 4B affinity resin. The procedure is described in the text. Elution with the buffer solution containing 1 mM 5'-adenylate started with fraction 23. The resin bed volume of the column was 100 ml and the volume of the fractions was 30 ml.

dure was dialyzed against buffer containing 0.05 M Tris– Cl, pH 7.2 and 0.5 mM DTT and was adsorbed onto a 2.5 × 20 cm column of the Compound III–Sepharose resin previously equilibrated with 0.02 M Tris–Cl pH 7.2, 0.2 M KCl, and 0.5 mM DTT and the enzyme was eluted by washing with the same buffer containing 1 mM AMP (fig. 2). The fractions having activity were pooled, concentrated in an Amicon ultrafiltration apparatus and dialyzed for 6 hr against 2 liters 0.05 M Tris–HCl buffer pH 7.2 containing 0.5 mM DTT and then for 12 hr against 500 ml of 50 mM Tris–Cl, pH 7.2, containing 2.5 mM DTT. The enzyme was stored at −70°C.

3. Results

3.1. *Chromatography of E. coli ADPglucose pyrophosphorylase*

ADPglucose pyrophosphorylase can be absorbed directly from crude extracts of *E. coli* cells by the affinity resin. It was found however that the capacity of the absorbant for the enzyme was dependent on the specific activity of the enzyme and increased if more purified enzyme preparations were used. The capacity is about 20 units/ml-packed resin for the activity in the ammonium sulfate fraction while for the activity in the DEAE-cellulose eluate it is at least 200 units/ml-packed resin. If the DEAE step is omitted and the ammonium sulfate step enzyme is applied directly to the column, a purification of 37-fold was obtained. This resulted in an enzyme solution with a specific activity of 70 units per mg-protein, and that showed one major band in gel electrophoresis with several minor bands.

Binding required no addition of substrates or Mg^{2+} to the enzyme solution. Elution of the enzyme was attempted with various solutions. Salt concentrations of 0.3 M KCl and above in 20 mM Tris–Cl, pH 7.2, buffer containing 2.5 mM DTT can elute the enzyme. If the above buffer solution contained 0.1 M KCl and 2 mM fru-P_2 the enzyme was eluted in a very large volume. This volume could be reduced by increasing the fru-P_2 concentration to 10 mM. The above buffer solution containing 0.1 M KCl plus 1 mM ATP or ADPglucose also eluted the enzyme in a very large volume. The enzyme could be eluted in a very small volume, usually less than the resin bed volume of the column with a 20 mM Tris–Cl, pH 7.2, solution containing 2.5 mM DTT, 0.1 M KCl and 1 mM 5'-adenylate (fig. 2).

This last solution was used for elution of the ADPglucose pyrophosphorylase from the affinity resin as described in the Materials and methods section. Table 1 summarizes the purification procedure and shows that a 6.3-fold purification of the DEAE-cellulose fraction resulted upon use of the affinity resin. The overall yield of the purification procedure was 52%; the yield from the affinity resin step was almost 90%.

The specific activity of the enzyme was 106 μmoles-min^{-1}-mg^{-1} and is the same as that obtained when the *E. coli* SG3 ADPglucose pyrophosphorylase was purified by a procedure using conven-

Table 1
Summary of purification of AC70R1 ADPglucose pyrophosphorylase

Fraction	Volume ml	Total protein mg	Total units μmoles-min^{-1}	Specific activity units-mg^{-1}
Crude extract	3000	121 000	33 400	0.28
Heat treatment	2700	37 500	32 672	0.87
Ammonium sulfate	550	11.000	25 100	2.3
DEAE-cellulose	89	1 148	19 400	16.9
Affinity resin	23	165	17 400	106

tional techniques, which involved 2–3 additional steps and gave only about a 25% yield [3]. Gel electrophoresis of 30 μg of protein in sodium dodecyl sulfate [8] showed one major band and a very faint and faster moving band when Coomassie Blue was used as the protein stain [19]. The subunit molecular weight was estimated to be 51,000. Gel electrophoresis of the enzyme in the Tris–glycine system [9] showed only one band which contained the enzyme activity as determined by an activity stain method [3].

Sucrose density gradient centrifugation [20] indicated that the molecular weight of the enzyme was 210,000. Thus, both subunit and native molecular weights of the enzyme were similar to those previously reported for the enzyme from *E. coli* SG3 [3].

4. Discussion

Sepharose derivatives covalently linked to competitive inhibitors or other substrate analogues have been frequently useful in the purification of enzymes [21]. In principle, any substance binding to a protein in a specific way can be used for affinity chromatography. Activators or inhibitors of allosteric enzymes should, therefore, not be overlooked as a potential basis of an affinity absorbent. 1,6-Hexanediol diphosphate which is an analog of fru-P_2 is also an activator of *E. coli* ADPglucose pyrophosphorylase with apparent affinity of the same magnitude was fru-P_2 [4]. The ability of 5′-adenylate to cause elution of the enzyme from the resin is consistent with this inhibitor's ability to counteract the activation caused by fru-P_2 [22].

It is expected that the absorbent used in this study can be applied for the purification of a number of enzymes that are activated or inhibited by fru-P_2. Unpublished results indicate rabbit muscle aldolase, in 0.15 M Tris–Cl, pH 7.2, buffer, can also be adsorbed onto the compound III–Sepharose resin. Rabbit muscle aldolase is competitively inhibited by hexanediol diP [12]. The aldolase can be eluted by using a 0.15 M Tris–Cl, pH 7.2, buffer solution containing either 1 mM fru-P_2 or 0.3 M KCl.

Other ADPglucose pyrophosphorylases having fru-P_2 as an allosteric effector [23] may also be highly purified using this affinity resin.

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