A RAPID PURIFICATION BY AFFINITY CHROMATOGRAPHY OF OROTATE PHOSPHORIBOSYLTRANSFERASE FROM *ESCHERICHIA COLI* K-12

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1. Introduction

The current interest in the role played by divalent cations in the orotate phosphoribosyltransferase (OPRTase)-catalyzed synthesis of orotidine-5′-monophosphate calls for further investigation to gain insight into the nature of the substrate–metal ions–protein interaction [1,2]. Physical methods such as NMR and ESR spectroscopy have proved to be valuable techniques in achieving this goal [1], but they need large amounts of protein. As the currently available purification procedures (requiring 9 steps) are rather tedious [3,4], we have elaborated a simple and rapid procedure based on affinity chromatography reported herein.

2. Materials and methods

5-Phosphoribosyl-β-D-ribose-diphosphate (PRPP) tetrasodium salt was purchased from Boehringer-Mannheim. Orotidine-5′-monophosphate was from Sigma. Orotic acid (Aldrich) was recrystallized from water. Cyanogen bromide was obtained from Merck, as were the chemicals for buffer solutions and $MgCl_2$ (which were analytical grade). Sepharose-4B was supplied by Pharmacia. Acrylamide, bis-acrylamide, *N,N,N,N′*-tetramethyl-ethylenediamine (Temed), ammonium persulfate and riboflavin were Bio-Rad electrophoresis quality products.

2.1. *Enzyme activity assays*

Enzyme activity was monitored by orotic acid disappearance at 20°C by following the decrease of absorbance at 291.5 nm [2]. Initial velocities were obtained from the slope of the absorbance variation within the first 30 s. The concentrations of reactants in 1 ml of assay solution were: 900 μM PRPP; 4 mM $MgCl_2$; 200 mM borate buffer (pH 9); 40–400 μM orotic acid.

2.2. *Non-denaturing polyacrylamide gel electrophoresis [5]*

The separating gels were prepared by mixing the various constituents as follows: 1 vol. solution (a) [48 ml 1 N HCl, 36.3 g Tris, 0.46 ml *N,N,N,N′*-tetramethylethylenediamine (Temed), H_2O to 100 ml]; 2 vol. solution (b) (30 g acrylamide, 0.8 g bis-acrylamide, H_2O to 100 ml); 1 vol. H_2O; 4 vol. solution (c) (140 mg ammonium persulfate in 100 ml H_2O). 7.5% gels were thus obtained.

The stacking gels were prepared as follows: 1 vol. solution (d) (25.6 ml 1 M H_3PO_4, 5.7 g Tris, H_2O to 100 ml, the pH is then 6.9); 2 vol. solution (e) (10 g acrylamide, 2.9 g bis-acrylamide, H_2O to 100 ml); 1 vol. riboflavin (4 mg/100 ml); 4 vol. H_2O; 10 μl 10 M dimethylaminopropionic acid.

The separating gels were poured in 0.5 cm i.d. glass tubes (1.5 ml) and stacking gels (0.3 ml) were poured on top. Upper and lower chamber buffers were obtained by mixing 6 g Tris, 26.8 g glycine in 1 liter of H_2O (pH 8.3). 150 μl of protein samples [50 μl protein preparation, 50 μl glycerol, 50 μl chamber buffer, 3 μl bromophenol blue (0.05% in Gly–Tris (pH 8.3) buffer)] were applied to the top of the stacking gel. A constant current of 4 mA/tube was applied. Gels were stained with Coomassie G-250 blue (0.04% in 3.5% perchloric acid) for 12 h and destained in 5% acetic acid. Unstained gels were sliced and assayed for activity.

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2.3. *Sodium dodecylsulfate (SDS) gel [6]*

Separating gel: 5.25 ml 40% acrylamide–0.6% bis-acrylamide, 2.6 ml Tris–HCl 1.5 M (pH 8.6), 4.5 μl Temed, 0.27 ml H_2O, 2 ml 40% sucrose, 0.2 ml 5% SDS, 20 μl 10% ammonium persulfate.

Stacking gel: 1.36 ml solution (c) (from non-denaturing gel preparation; cf. above), 2.5 ml Tris–HCl 0.5 M (pH 6.8), 12 μl Temed, 5.88 ml H_2O, 200 μl 5% SDS, 30 μl 10% ammonium persulfate.

Chamber buffer: 6.9 g Tris, 26.8 g glycine, 0.1% SDS in 1 liter of H_2O.

Sample buffer: 0.1% SDS, 0.01 M sodium phosphate (pH 7.2), 0.14 M β-mercaptoethanol, 10% (in volume) glycerol, 0.002 M bromophenol blue.

Protein samples were prepared by heating 5–100 μl of protein solutions in 100 μl of sample buffer at 65°C for 10 min. The following protein standards were used: bovine serum albumin (1.3 mg/ml), creatine kinase (1.5 mg/ml), lactate dehydrogenase (1.5 mg/ml).

2.4. *Synthesis of OMP–Sepharose*

Sepharose 4B was activated by cyanogen bromide according to [7] and was coupled to adipic acid dehydrazide as in [8].

5′-OMP (23 μmol) were added to 25 μmol sodium *meta*-periodate in 1 ml cold 100 mM phosphate buffer (pH 7.4). The reaction was allowed to proceed for 2 h in the dark at 4°C.

Centrifuged hydrazide–Sepharose (16 ml) was washed 3 times with 10 ml 100 mM acetate buffer (pH 5).

Oxidized (1 ml) OMP was added to 16 ml centrifuged hydrazide–Sepharose in 4 ml acetate buffer (total vol. 21 ml). Aliquots (2 ml) were pipetted every 30 min and centrifuged. The concentration of unfixed oxidized 5′-OMP was measured from the UV spectrum of the supernatant. After 2 h no absorbance was detected in the supernatant.

NaCl (3 ml, 3 M) was added to the 21 ml solution. No release of OMP was observed, thereby suggesting that specific covalent fixation rather than non-covalent binding to the hydrazide–Sepharose gel occurred. 1.4 μmol oxidized OMP were retained/ml packed gel.

OMP–Sepharose (16 ml) were packed in a 15 mm i.d. column to a height of 10 cm. The column was thoroughly washed with 100 mM sodium phosphate (pH 7.2) and stored at 4°C.

3. Purification of OPRTase from *E. coli*

Escherichia coli (K-12 strain, 1 g/ml) disrupted in a French press (32 000 lb/in^2) were suspended in 50 mM potassium phosphate (pH 7.5) and centrifuged (10 000 × *g*) for 30 min. The supernatant was centrifuged again at 170 000 × *g* for 2 h (solution I).

3.1. *Step 1: Thermal denaturation*

$(NH_4)_2SO_4$ (20 ml, 3.6 M) were added to 75 ml solution I. The mixture was heated at 75°C for 3 min and centrifuged (table 1).

Table 1
Purification of *Escherichia coli* K-12 OPRTase

	Protein (mg/ml)	Specific OPRTase activity[a,b]	Total[c] activity	Yield
Crude extract	5.5	1.42×10^{-2}	5.8	–
Thermal denaturation and ammonium sulfate fractionation	1.3	10^{-1}	1.3	0.23
Affinity chromatography	0.043	27	1.3	≃1

[a] Activity in μmol consumed orotate . mg protein^{-1} . min^{-1}
[b] For assay conditions cf. section 2
[c] Total activity in μmol consumed orotate/min

3.2. *Step 2: Ammonium sulfate fractionation*

Supernatant (85 ml) from step 1 (solution II) was dialyzed against 170 ml 3.6 M $(NH_4)_2SO_4$ at 4°C for 6 h. No OPRTase activity was detected in the supernatant after ammonium sulfate precipitation. The precipitate was dissolved in 6 ml 100 mM sodium phosphate buffer (pH 7.2). OMP-decarboxylase, together with OPRTase, was present in this solution.

3.3. *Step 3: Affinity chromatography*

The enzyme solution from the preceding step (6 ml) was poured on top of the affinity column and was allowed to enter the gel at 20 ml/h. The column was then washed with 75 ml 100 mM NaH_2PO_4 buffer at pH 7.2, and subsequently with 75 ml 10 mM Na_2HPO_4 at pH 8.2 at 80 ml/h.

The adsorbed proteins were then eluted either with 3 ml of a 17 mM PRPP and 17 mM $MgCl_2$ solution or with a 4 mM orotate and 4 mM $MgCl_2$ solution in 10 mM Na_2HPO_4 buffer at pH 8.2. The elution rate was 20 ml/h. The enzymatic activity was quantitatively recovered. The elution profile (fig.1) displays a very sharp peak so that both purification and concentration of the protein were thus achieved without further treatment.

No OMP decarboxylase activity was detected in the eluent. The affinity column retains its efficiency even after numereous runs.

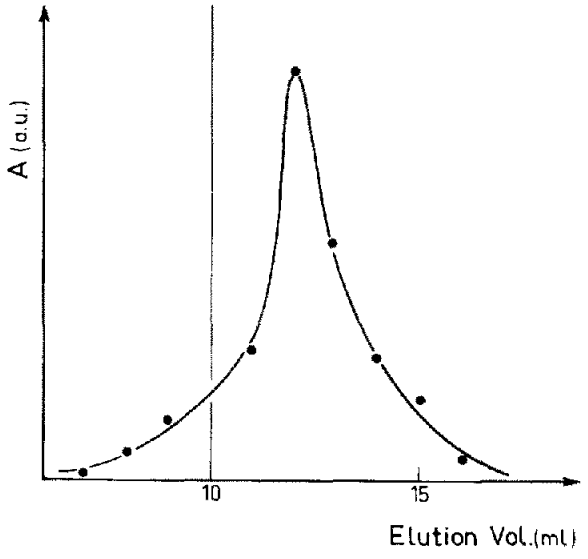

Fig.1. Elution of OPRTase activity from OMP–Sepharose affinity gel.

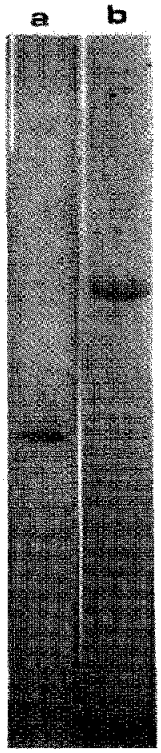

Fig.2. (a) Non-denaturing gel electrophoresis of OPRTase purified by OMP–Sepharose affinity chromatography. (b) SDS gel electrophoresis (cf. section 2) samples of 2 μg purified protein.

The protein was homogeneous by the criterion of nondenaturing gel electrophoresis (fig.2). Slices 3 mm thick of unstained gel were introduced into aliquots of the assay mixture. OPRTase activity was observed at an R_F identical to that of the protein band on the stained gel. SDS gels showed one band (fig.2). Calibration of the gels with known proteins (cf. section 2) led to an estimate of the subunit M_r at 22 000 (± 2000).

The purified protein solution mixed with an equal volume of glycerol in the presence of 10^{-4} M dithiothreitol was kept at −25°C. A certain amount of decomposition occurred with time, as shown by a loss of activity (35% in 40 days) accompanied by the appearance of new bands at the top of the acrylamide gels.

4. Discussion

The OMP–Sepharose gel has proved to be well-

suited to the purification of OPRTase from bacteria. It is expected that OPRTase from other organisms could be purified as well. Indeed we were able to prepare pure baker's yeast OPRTase (from an OMP-decarboxylase/OPRTase mixture supplied by Sigma) using our affinity column.

Under the conditions of the elution, the OMP decarboxylase has no (or only slight) decarboxylating activity at the gel-bound OMP. Specific elution of OPRTase by the normal PRPP or orotic acid enzyme substrate, together with the non-retention of OPRTase on hydrazide–Sepharose and UMP–Sepharose gels, strongly suggests that what occurs is true biospecific fixation of the enzyme to the immobilized ligand, rather than non-specific hydrophobic bonding.

Elution of the immobilized protein by orotate or PRPP in the presence of Mg^{2+} shows that both ligands have a good affinity for the OMP–protein complex. Substrate interactions with the bound Enz–OMP complex are represented on following scheme:

Sloan has postulated that yeast OPRTase catalyzed OMP synthesis proceeds via an ordered mechanism involving the addition of PRPP to the protein and pyrophosphate formation in a first step followed, in a second step, by orotate fixation to the phosphoribosylated protein [4]. According to this hypothesis step II_3 is balanced and step II_3' is tentatively included in proceeding scheme.

The fixation of orotate to (Enz–OMP) (shown here) and to (Enz–PR) [4] suggests that the ribosyl-5′-phosphate group is essential to yield enzyme complexes possessing high affinity towards orotate.

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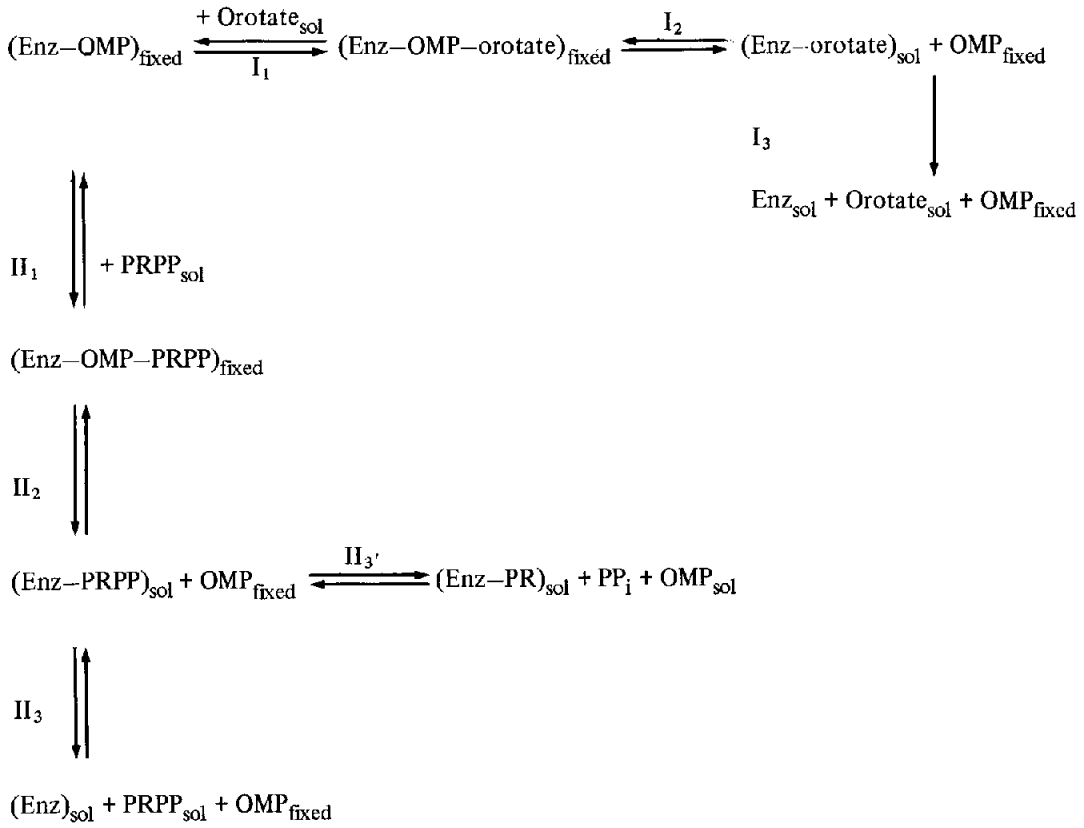

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