Availability of GTP-Sepharose for the Purification of Glutamate Dehydrogenase from Eel Liver

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(Received July 12, 1976)

Glutamate dehydrogenase from eel liver was purified 75.5 fold in 33% yield by ammonium sulfate fractionation, protamine and heat treatment, gel filtration and affinity chromatography on GTP-Sepharose. When the partially purified enzyme from gel filtration was applied to a GTP-Sepharose column, it was retained with very few other proteins and recovered in 80 to 90% yield.

The high affinity for ADP of glutamate dehydrogenase from eel liver was applied to the purification of this enzyme by affinity chromatography. Although this enzyme was retained on the ADP-Sepharose column, it could not be eluted either by a KCl gradient or by a pulse of NADH.

Glutamate dehydrogenase (EC 1.4.1.2) is a key enzyme in ammonia metabolism and has been studied extensively by numerous authors^{1,2,3)}. In liver of eel, *Anguilla japonica*, it is supposed that glutamate dehydrogenase plays an important role in the regulation of nitrogen metabolism. However very little is known about this aspect and the enzyme.⁴⁾

This paper reports a method for the purification of glutamate dehydrogenase from eel liver by GTP-Sepharose. The use of affinity chromatography for the selective binding of specific proteins has proved extremely useful for the purification of a number of enzymes⁵⁾. Glutamate dehydrogenase from eel liver, like the enzyme from other sources, has the high affinity for GTP which is a powerful inhibitor.⁶⁾ We describe that the affinity chromatography on GTP-Sepharose was useful for the purification of this enzyme.

Experimental Procedures

Materials L-Glutamic acid, α -ketoglutaric acid and protamine sulfate were purchased from Wako Chemicals Co. NAD⁺ and NADH were obtained from Oriental Yeast Co. Sephadex G-150 and CNBr-activated Sepharose were the product of Pharmacia. GTP, ATP, ADP and AMP were obtained from Yamasa Soybean Souce Co. CTP and ITP were purchased from Sigma Chemical Co. L-glutamate γ -methylester was purchased from Protein Research Foundation. Other chemicals were obtained from standard commercial sources.

Preparation of acetone powder from eel livers The acetone powder of eel livers was

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prepared essentially as described by L. CORMAN *et al.*⁷⁾ When the livers were homogenized in distilled water or appropriate buffers, difficulties attributable to the excessive quantities of oil and fat were encountered. These obstacles were neatly obviated by resort to an acetone powder. A suitable quantity of minced eel livers was homogenized with approximately 10 volumes of acetone (-20°C) by Polytron homogenizer. The homogenate was filtered and the precipitate was suspended and rehomogenized in 5 volumes of *n*-butanol (-20°C). After filtration, the residue was again extracted with a few volumes of acetone and refiltered. The precipitate was then washed with 2 volumes of anhydrous ether (-20°C). The leached "cake" was raked together and dried at room temperature under reduced pressure.

Extraction The acetone powder was extracted with 10 volumes of 0.1 M Tris-HCl buffer (pH 8.0) at room temperature for 1 hr.

Preparation of GTP-Sepharose The GTP-Sepharose was prepared essentially according to R. J. JACKSON *et al.*⁸⁾ and C. GODINOT *et al.*⁹⁾ CNBr-activated Sepharose, 5 g, was suspended in 100 ml of cold 1 mM HCl for 10 min, filtered with glass filter and washed with 900 ml of cold 1 mM HCl. The gel was washed with 1.5 l of water and bufferized with 1 l of 0.1 M NaHCO_3 buffer (pH 9.0). Suspension of the gel, 50 ml, and 1.5 g of L-glutamate γ -methylester in 20 ml of 0.1 M NaHCO_3 buffer (adjusted to pH 9.0 with 1 N NaOH) was mixed. The mixture was shaken at room temperature for 2 hr and allowed to stand for about 1 to 2 hr. The gel was washed with 1.5 l of water. Hydrazine hydrate (85%), 59 ml, in flask (300 ml) was preincubated at 70°C for about 10 min. Then L-glutamate γ -methylester-Sepharose was added dropwise to the hydrazine hydrate and incubated at 70°C for 15 min. After incubation the hydrazide gel was cooled to room temperature and washed with 1.5 l of water.

On the other hand 90 μmoles of GTP in 10 ml of 0.1 M citrate phosphate buffer (pH 5.0) was oxidized by 1.0 ml of 0.1 M periodate at room temperature for 30 min in the dark. To destroy excess periodate, 0.4 ml of ethyleneglycol (0.22%) was added and the mixture was oxidized an additional 10 min.

Periodate oxidized GTP and hydrazide-Sepharose were mixed and shaken for 2 hr at room temperature. To block the residual hydrazide, 0.06 ml of formaldehyde (37%) was added to the mixture and the mixture was shaken an additional 1 hr.

Then the gel was washed with 1 l of cold 0.1 M phosphate buffer-5 mM EDTA (pH 6.8), with 1 l of cold 2 M KCl and with 1.5 l of cold water and bufferized with 1.5 l of cold 0.05 M Tris-HCl buffer-1 mM EDTA (pH 7.5).

The coupling yield of L-glutamate γ -methylester to Sepharose was determined by colorimetric method using ninhydrin reagent. The yield of covalent binding of GTP to the hydrazide-Sepharose was determined by measuring absorbance at 253 $\text{m}\mu$.

Preparation of ADP-Sepharose ADP-Sepharose was prepared according to the pro-

cedures described in "Preparation of GTP-Sepharose" except that ADP were used as a ligand instead of GTP. The yield of covalent binding of ADP was determined by measuring absorbance at 260 m μ .

Enzyme assays and protein determination In case of reductive amination the assay mixture (4.0 ml) consisted of 50 mM NH₄Cl, 20 mM α -ketoglutarate (adjusted to pH 8.0 with 1 M KOH), 0.15 mM NADH and 20 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM EDTA. The reaction was initiated by the addition of 50 μ l of enzyme solution. The oxidation of NADH at 30°C was followed by the decrease in absorbance at 340 m μ with a Hitachi EPU-2A. One enzyme unit was defined as the amount of enzyme that catalyzed the conversion of 1 μ mole of NADH per min under the assay conditions.

In case of oxidative deamination the assay mixture (4.0 ml) consisted of 0.1 M Tris-HCl buffer (pH 8.0), 0.1 M glutamate and 5 mM NAD⁺. Activity was measured by following the increase in absorbance at 340 m μ .

Protein was determined by the procedure of LOWRY *et al.*¹⁰⁾ or from absorbance at 280 m μ .

Results

A representative procedure is described below for the supernatant solution extracted from the acetone powder obtained from 400 g (wet weight) of eel livers.

Ammonium sulfate fraction The supernatant solution was brought to 25% saturation by slow addition of solid (NH₄)₂SO₄ with continuous stirring. After 20 hr the suspension was centrifuged at 15,000 $\times g$ for 30 min and the supernatant was taken to 60% saturation in (NH₄)₂SO₄. After 20 hr the suspension was centrifuged as above. The pellet contained most of the enzyme. The pellet was dissolved in a few volumes of 0.1 M Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and dialyzed against 0.01 M Tris-HCl buffer-0.1 mM EDTA (pH 8.0).

Protamine precipitation of nucleoprotein and heat treatment Protamine sulfate was dissolved in the dialyzed solution at a concentration of 10.75 mg per 100 mg of protein. After 20 min, the precipitate was removed by centrifugation for 30 min at 15,000 $\times g$. The supernatant solution was heated at 55°C for 8 min with constant stirring. The precipitate was removed by centrifugation. Then the supernatant was concentrated on a XM 50 Amicon ultrafiltration membrane.

Gel filtration The concentrated solution was applied to a column (2.6 $\times$ 90 cm) of Sephadex G-150 previously equilibrated with 0.01 M Tris-HCl buffer-0.1 mM EDTA (pH 8.0) containing 0.1 M NaCl. The enzyme was eluted with the same buffer at a flow rate of 15 ml per hr and fractions of 5 ml were collected as shown in Fig. 1. Fractions with high specific activity were pooled, concentrated on a XM 50 Amicon ultrafiltration membrane and dialyzed against 0.05 M Tris-HCl buffer-1 mM EDTA (pH 7.5).

Fig. 1. Gel filtration of glutamate dehydrogenase in 0.01 M Tris-HCl buffer-0.1 mM EDTA-0.1 M NaCl at pH 8.0 on a column (2.4×90 cm) of Sephadex G-150.

Affinity chromatography on GTP-Sepharose column The results of experiments for the preparation of GTP-Sepharose indicated that about $130 \mu\text{moles}$ of L-glutamate γ -methylester as a spacer were coupled per ml of settled gel and approximately $2 \mu\text{moles}$ of oxidized GTP were bound per ml of settled gel. However the release of phosphates of GTP was not taken into consideration for the determination of the coupling yield of GTP. The UV-spectrum of GTP-Sepharose was shown in Fig. 2.

Fig. 2. UV-spectrum of GTP-Sepharose. GTP-Sepharose was suspended in 0.05 M Tris-HCl buffer (pH 7.5) containing 1 mM EDTA.

Glutamate dehydrogenase fraction separated by gel filtration was applied to a column (1.6×14.5 cm) of GTP-Sepharose previously equilibrated with 0.05 M Tris-HCl buffer (pH 7.5) containing 1 mM EDTA. After washing the column with 75 ml of the equilibrated buffer, the enzyme was eluted with a linear gradient of KCl, formed by placing 100 ml of the equilibrated buffer in the mixing chamber and 100 ml of the same buffer containing 1 M KCl in the reservoir. Fractions of 5 ml were collected at a flow rate of 20 ml per hr. The enzyme was eluted at approximately 0.1 M KCl. A typical elution profile is shown in Fig. 3.

Fig. 3. Chromatography of glutamate dehydrogenase on GTP-Sepharose. Enzyme protein (18.56 mg) in 0.05 M Tris-HCl buffer-1 mM EDTA (pH 7.5) was applied to a 1.6×14.5 cm column, equilibrated with the same buffer.

Table 1. Summary of the results of the purification

Procedure	Protein mg	Total Units	S. A. (units/mg protein)	Recovery %
25-60% $(\text{NH}_4)_2\text{SO}_4$	4814	2147	0.45	100
Protamine and Heat treatment	1681	1731	1.03	81
Sephadex G-150	184	787	4.28	37
GTP-Sepharose	21	708	33.79	33

The purification procedure is summarized in Table 1.

Affinity chromatography on ADP-Sepharose column ADP-Sepharose prepared indicated that approximately $2 \mu\text{moles}$ of oxidized ADP were bound per ml of settled gel. The enzyme partially purified by gel filtration was applied to a column (1.6×14 cm) of ADP-Sepharose previously equilibrated with 0.05 M Tris-HCl buffer (pH 7.5) containing 1 mM EDTA. Though the enzyme was retained on the ADP-Sepharose column, it could not be eluted either by a KCl gradient or by addition of 1 ml of 1 mM NADH.

Discussion

Glutamate dehydrogenase from eel liver was purified 75.5 fold with a yield of 33% by our procedure including affinity chromatography on the GTP-Sepharose. When the enzyme partially purified by the gel filtration was applied to the column of the GTP-Sepharose, it was retained with very few other proteins and the recovery of enzyme activity from the column ranged from 80 to 90%. The specific activity of the enzyme eluted from the column was 33.8 units per mg protein. However polyacrylamide gel electrophoresis of the concentrated eluate revealed the enzyme was not homogeneous.

About 3 units of the glutamate dehydrogenase were bound per ml of GTP-Sepharose gel. This retention capacity was about the half of that reported by C. GODINOT *et al.*⁹⁾

Since L-glutamate γ -methylester could not become the substrate for the oxidative deamination instead of L-glutamate and did not inhibit the reductive amination, it was assumed that there was no possibility of L-glutamate γ -methylester residue of the gel to be a ligand.

It was found from the effects of nucleotides on the glutamate dehydrogenase partially purified from eel liver that ADP strikingly accelerated NAD^+ reduction and NADH oxidation. Then the purification of the enzyme by the affinity chromatography on ADP-Sepharose was examined. Although the enzyme was retained on the ADP-Sepharose column, it could not be eluted either by a KCl gradient or by a pulse of NADH. It is assumed that the affinity for ADP of the enzyme is stronger than for GTP or that the mechanism of the interaction between the enzyme and immobilized GTP is different from it between the enzyme and immobilized ADP.

Affinity chromatography on immobilized adenosine-5'-monophosphate has been utilized to separate and purify dehydrogenases and kinases^{11,12)}. It is thought that 5'-AMP is a suitable ligand for affinity chromatography since it is an inhibitor for a great number of both NAD^+ -dependent and other enzymes. However the glutamate dehydrogenase from eel liver is not inhibited by 5'-AMP. Therefore it is assumed that AMP-Sepharose is not useful for the purification of the enzyme.

BLUMENTHAL *et al.*¹³⁾ reported purification of *Neurospora* glutamate dehydrogenase by affinity chromatography on a resin containing N-carboxymethyl-L-glutamate but elution of the enzyme could be obtained only with an acidic buffer that induced partial inactivation.

From the present investigation it may be concluded that the affinity chromatography on GTP-Sepharose was very useful for the purification of the glutamate dehydrogenase from eel liver.

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