PURIFICATION OF THIAMINE-BINDING PROTEIN FROM ESCHERICHIA COLI BY AFFINITY CHROMATOGRAPHY

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SUMMARY

An affinity column coupled with thiamine pyrophosphate quantitatively absorbs the thiamine-binding activity from a partially purified preparation of Escherichia coli. The thiamine-binding protein can be eluted from the affinity column in high yield by use of 8 M urea-containing buffer. Approximately 90-fold purification occurs by affinity chromatography yielding a preparation which appears to be homogenous.

Recent studies (1, 2) have shown that Escherichia coli contains a thiamine-binding protein which is thought to be concerned with the transport of thiamine through the cell membrane. The properties of a partially purified thiamine-binding protein from E. coli have been reported (1). However, it has not yet been purified enough to investigate its physical properties.

During an investigation into the properties of the thiamine-binding protein from E. coli, we found that the partially purified protein can bind to thiamine phosphates such as thiamine pyrophosphate (TPP) and thiamine monophosphate (TMP), as well as thiamine (3).

In the present paper we describe that an affinity chromatography using agarose coupled with TPP is potentially useful for purification of the thiamine-binding protein from E. coli, and some of physical properties of the purified protein.

MATERIALS AND METHODS

Sepharose 6B was obtained from Pharmacia, cyanogen bromide from Wako Pure

Chemical Industries, TPP from Sigma Chemical Company and ^{14}C-thiamine (thiazole-2-^{14}C), specific activity 14.0 mCi/mmole, from Radiochemical Centre, England.

Sepharose 6B (50 ml) was activated with cyanogen bromide (6 g) at pH 11 for 1 hr at 25°, filtered and washed rapidly on the funnel with 2 liters of cold water and 1 liter of cold 0.1 M $NaHCO_3$. The damp gel was resuspended in 100 ml of cold 0.1 M $NaHCO_3$ containing 6 g of ethylenediamine and the pH of this solution was adjusted to 10. The suspension was stirred for 11 hr at 4°, and it was filtered, washed with 3 liters of cold water. The resulting ω-aminoethyl derivative of Sepharose was reacted with 500 mg of TPP and 0.6 ml of 1-ethyl-3-(3-dimethylaminoprophyl) carbodiimide at pH 6.4. After reaction for 16 hr at 4°, the gel was washed with a large volume of cold water and the final product was stored as an aqueous suspension in the cold. The amount of TPP bound to Sepharose was estimated to be 38.8 nmoles per ml resin by the manometric method (4).

Activity of thiamine-binding protein was assayed using equilibrium dialysis as previously described (2). The specific activity was expressed as the amount of thiamine bound per mg of protein. The protein was measured by the procedure of Lowry et al. (5).

RESULTS AND DISCUSSION

A mutant of E. coli K12 auxotrophic for thiamine thiazole (KG 33) was grown in 90 liters of the minimal medium of Davis and Mingioli (6) containing 0.2 % glucose and 0.04 μM thiamine thiazole. They were harvested at the late exponential phase and the cells were then subjected to the cold osmotic shock according to the procedure of Neu and Heppel (7). The shock fluid (4.5 liters) was concentrated to 940 ml using Diafilter MC-4 ultrafiltration apparatus (Ulvac Corporation, Kanagawa, Japan) under N_2 pressure at 4°. The concentrated shock fluid was fractionated with ammonium sulfate between 65 and 95 % saturation, and the precipitated protein was recovered by centrifugation at 10,000 x g for 20 min, dissolved in 60 ml of 0.05 M K-phosphate buffer, pH 7.0, and dialyzed

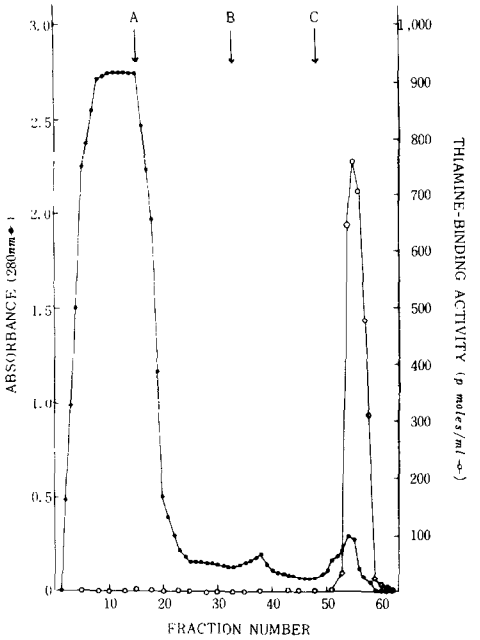

Fig. 1.

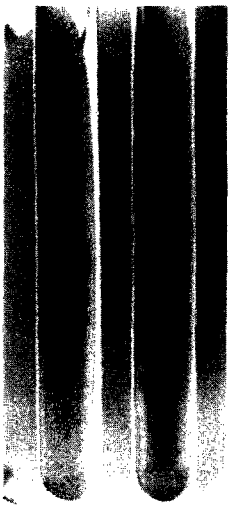

Fig. 2.

Fig. 1. Affinity chromatography of partially purified thiamine-binding protein on TPP-Sepharose column. The column (1.4 X 8 cm) was equilibrated with 0.05 M K-phosphate buffer, pH 7.0, and 29 ml of Step 3 was applied to the column. At the designed intervals the following reagents were introduced into the column : (A) 0.05 M K-phosphate buffer, pH 7.0, (B) 4 M urea in 0.05 M K-phosphate buffer, pH 7.0, (C) 8 M urea in 0.05 M K-phosphate buffer, pH 7.0. Two ml fractions were collected. The flow rate was about 4 ml per hour, and the experiment was performed at 4°.

Fig. 2. Polyacrylamide gel electrophoresis of purified thiamine-binding protein. The gels contained 15 % acrylamide, 0.4 % bisacrylamide and 0.75 M Tris-HCl, pH 8.9. The amount of protein applied was 75 µg and the electrophoresis was run at 4 ma per tube for 2 hr (A) and 5 hr (B). Protein migrated to the anode (bottom) and was stained in amido black 10B.

against 3 liters of 0.05 M Tris-HCl, pH 8.0, with two changes of buffer. Following dialysis, the solution contained 340 mg of protein in 80 ml was applied to DEAE-cellulose column (2.4 X 18 cm) which was previously equilibrated with 0.05 M Tris-HCl, pH 8.0, and washed with 270 ml of the same buffer. The washing was concentrated to 29 ml by ultrafiltration and then applied to the column of TPP-Sepharose (1.4 X 8 cm)

Virtually all of the thiamine-binding activity from a partially purified preparation from E. coli KG 33 was adsorbed to the column (Fig. 1). The

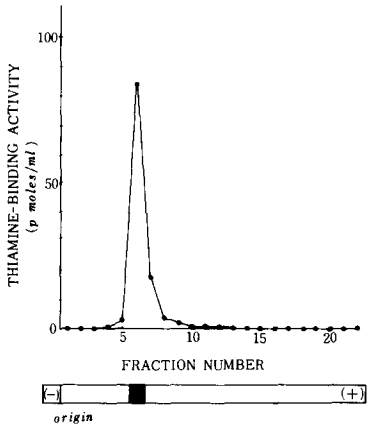

Fig. 3. Binding activity of the thiamine-binding protein during polyacrylamide gel electrophoresis. The nonstained part of the gel described in Fig. 2 (A), which was run in duplicate, was cut in the parallel pieces (4 mm in length) along the direction of migration. The gel pieces were numbered from the origin to the anode and homogenized in 1.4 ml of 0.05 M K-phosphate buffer, pH 7.0. One ml each of the extracts was used for the assay of thiamine-binding activity.

binding of thiamine-binding protein to the column was so strong that elution did not occur with 4 M urea. With 8 M urea in 0.05 M K-phosphate buffer, pH 7.0, the thiamine-binding protein was quantitatively eluted. The protein denaturated with 8 M urea is completely reconstituted by dialysis to lower the concentration of urea (1).

As shown in Table I the specific activity of the purified protein represents an overall purification of approximately 90-fold with a total recovery of 23.2 %. The product exhibits a single protein band in standard polyacrylamide gel electro phoresis (Fig. 2) and the binding activity toward thiamine was coincident with the stained part of the gel (Fig. 3).

The molecular weight of the thiamine-binding protein was found to be 39,000 by the measurement of elution volume from Sephadex (Fig. 4).

TMP also couples to aminoethyl-Sepharose, whereas free thiamine doesn't under the condition used. TMP-Sepharose, however, has no significant ability to bind thiamine-binding protein from E. coli. Although the mode of coupling between thiamine phosphates and agarose has been unknown, there must be some steric interference with the binding process in the case of TMP-Sepharose.

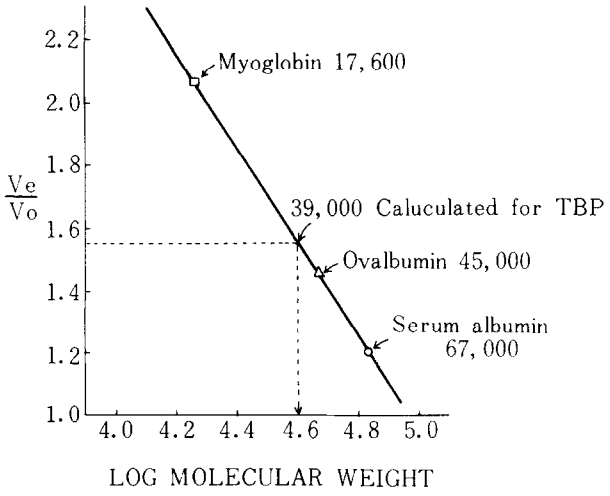

Fig. 4. Molecular weight determination by Sephadex G-100 column chromatography. The purified thiamine-binding protein (0.2 mg) was applied to the column of Sephadex G-100 (1.5 X 90 cm). The column was eluted with 0.01 M Tris-HCl, pH 7.5, at room temperature and 1.0 ml fractions were collected. Thiamine-binding activity was determined by equilibrium dialysis. The ratio of elution volumn (Ve) to void volume (Vo) was plotted against the log of the molecular weight for each known protein.

TABLE I

Purification of thiamine-binding protein from E. coli KG 33

Step	Total activity	Total protein	Specific activity
	p moles	mg	p moles/mg
1. Shock fluid	33,946	630	53.9
2. Ammonium sulfate, fraction 65 to 95 %	10,506	346	30.4
3. Negative adsorption on DEAE-cellulose	7,947	43	184
4. Affinity chromatography	7,880	1.6	4,925

Attempt to adsorb the binding activity on TPP-Sepharose directly from a concentrated shock fluid from E. coli KG 33 was also successful. The protein was purified approximately 30-fold with a yield of 50 to 60 %, in a single step.

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