PURIFICATION OF THIAMINE-BINDING PROTEIN OF RAT ERYTHROCYTES BY AFFINITY CHROMATOGRAPHY

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Several proteins which specifically bind vitamins have now been isolated and studied [3, 9, 11, 12]. The role of these proteins in metabolism in vivo is determined by the function of transport of vitamins through biological membranes or their temporary retention inside cells and subcellular formations.

A protein binding thiamine has been isolated from various objects and has been studied in the greatest detail in microorganisms [4, 8] and yeast cells [5]. The presence of such a protein in higher organisms has been demonstrated only in hens' eggs [6].

The erythrocytes of animals are a convenient cell model and, at the same time, they possess considerable activity of thiamine-dependent enzymes. Accordingly these blood cells were chosen as a suitable test object.

EXPERIMENTAL METHOD

The protein was isolated and purified by affinity chromatography of a hemolysate of erythrocytes on sorbents with no cationic charge at the point of attachment of the ligand to the carrier and differing in their degree of hydrophobicity: a) thiamine-N-4-azobenzoyl-ε-aminocaproyl hydrazine-sepharose, b) thiamine-N-4-azobenzoyl-glycylglycine hydrazide-sepharose. The conditions of synthesis of the affinity matrices were described previously [1].

Erythrocytes were obtained and hemolyzed in accordance with the technical instructions in [2].

Binding of the protein with the sorbent was achieved by mixing the erythrocyte hemolysate and affinity resin for 30 min at 4°C and pH 7.0, in medium containing Mg^{++} ions (0.1 mM). This was followed by washing with 0.02 M Tris-HCl, pH 7.0, to remove ballast proteins. Stepwise gradient elution was carried out with buffer of the same molarity, at pH 8.0 and 9.0. The samples were dialyzed against 0.04 M Tris-HCl, pH 7.0 (1:500 ml). Protein was determined by Lowry's method [7] and tested for thiamine-binding activity by incubation with radioactive [thiazole-2-^{14}C]-thiamine for 30 min at 37°C and pH 7.0. Bound and free label were separated on a column with Sephadex G-25. Radioactivity was measured on an LKB "Wallace" (Sweden) liquid scintillation counter. Specific activity was expressed as the number of moles of thiamine bound by 1 mg protein under the conditions mentioned above [5].

EXPERIMENTAL RESULTS

Chromatography of the erythrocyte hemolysate, as shown in Fig. 1, demonstrates that the thiamine-binding activity was associated with protein peak B. Peaks A, C, and D did not possess such activity. These proteins are evidently not speficially bound with the sorbent by hydrophobic interactions. The results of experiments on equilibrium dialysis and gel-chromatography indicate that binding between thiamine and protein is sufficiently firm within the pH range 6.8-7.2. Dialysis in a buffer system under more alkaline conditions leads to detachment of the label. The fact that Mg^{++} and Mn^{++} ions influence binding is evidence that thiamine binds with protein only in the form of such a complex.

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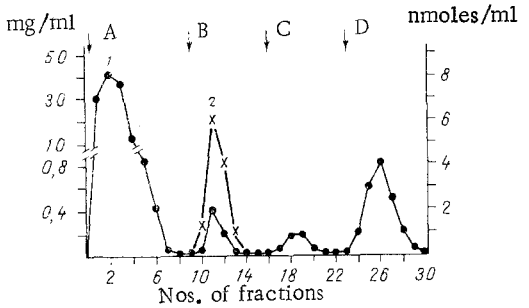

Fig. 1. Affinity chromatography of erythrocyte hemolysate. A) Elution with 0.02 M Tris-HCl, pH 7.0; B) elution with 0.02 M Tris-HCl, pH 8.0; C) elution with 0.02 M Tris-HCl, pH 9.0; D) elution with 8 M urea. 1) Protein profile, 2) thiamine-binding activity.

TABLE 1. Purification of Thiamine-Binding Protein from Rat Erythrocytes by Affinity Chromatography

Stage of purification	Total protein	Total thiamine-binding activity, nmole	Specific activity, nmoles/mg	Yield, %
Erythrocyte hemolysate	9900	62.37	0.006	-
Affinity chromatography	2.29	31.42	13.72	50

The data in Table 1 show that the suggested method can yield a preparation of thiamine-binding protein with specific activity of 13.72 nmoles/mg. The degree of purification was 2300 times and the yield 50%. The protein preparation was homogeneous on electrophoresis in polyacrylamide gel in the presence of sodium dodecylsulfate. Similar protein preparations isolated from yeast [5] and from *E. coli* [10], it must be pointed out, had rather lower specific activity (63.4 pmoles/mg and 4.2 nmoles/mg respectively), yet the authors cited used five or six somewhat laborious methods of purification.

The more detailed study of the kinetics of binding and dissociation of the thiamine–protein complex and determination of the role of metals in this process will help to shed light on the function of thiamine-binding protein in vitamin B_1 transport in animal erythrocytes.

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