IMMOBILIZED DERIVATIVE OF PYRIDOXAL 5'-PHOSPHATE. APPLICATION TO AFFINITY CHROMATOGRAPHY OF TRYPTOPHANASE AND TYROSINE PHENOL-LYASE

Sei-ichiro IKEDA, Hitoshi HARA, Susumu SUGIMOTO and Saburo FUKUI*

Laboratory of Industrial Biochemistry, Department of Industrial Chemistry, Faculty of Engineering, Kyoto University, Sakyo-Ku, Kyoto, Japan

Received 9 June 1975

1. Introduction

Hitherto we have prepared three kinds of immobilized derivatives of pyridoxal-P**: 6-immobilized, N-immobilized and 3-O-immobilized analogues bound covalently to Sepharose-4B matrix at the indicated position [1,2]. Since all of these Sepharose-bound pyridoxal-P possess free 4-formyl groups and 5'-phosphate groups which are essential for binding with apoproteins of various vitamin B_6 enzymes, these compounds are expected to be useful for affinity chromatography and studies on the coenzyme binding properties of vitamin B_6 enzymes. Immobilization of some vitamin B_6 enzymes consisting of multi-subunits, such as tryptophanase, was successfully achieved through the specific binding of immobilized pyridoxal-P to the lysine residue at the active site of the one subunit [1–4].

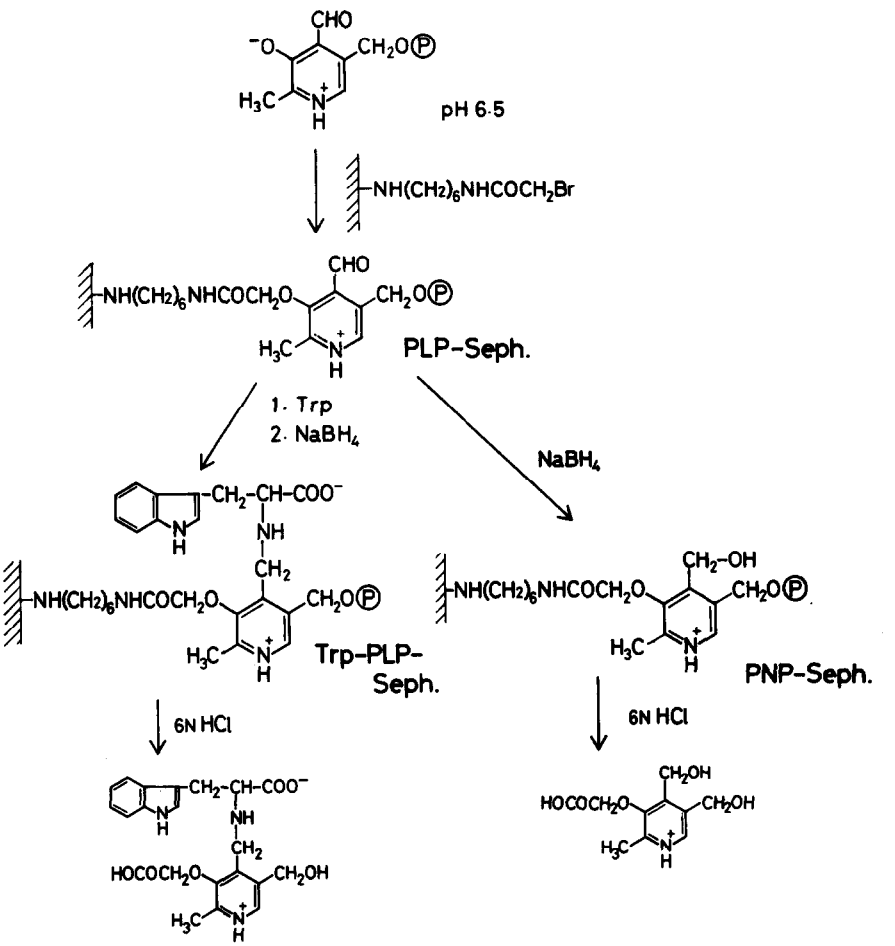
In the present paper we describe the application of one of the abovementioned immobilized derivatives of pyridoxal-P, i. e., 3-O-immobilized pyridoxal-P (referred to as PLP-Seph.) for the affinity chromatography of apotryptophanase from *Escherichia coli*. The enzyme was efficiently adsorbed on a column of PLP-Seph. and eluted selectively with a mixture of 0.5 M potassium phosphate buffer and 0.5 mM pyridoxal-P. Moreover, PLP-Seph. served as an excellent biospecific adsorbent for apo-tyrosine phenol-lyase requiring pyridoxal-P. These results indicate that Sepharose-bound pyridoxal-P such as PLP-Seph. would be very useful as general ligands for the affinity chromatography of various vitamin B_6 enzymes. Furthermore, a complex of PLP-Seph. and tryptophan (Trp-PLP-Seph.) [2] was found to be a more specific adsorbent for apo-tryptophanase. In this case, apo-tryptophanase adsorbed on a column of Trp-PLP-Seph. was specifically eluted with a mixture of KPB, pyridoxal-P and tryptophan. Such an immobilized complex of pyridoxal-P and an appropriate amino acid would be useful for the affinity chromatography of a pyridoxal-P dependent enzyme involved in the metabolism of the amino acid. Such an immobilized complex of pyridoxal-P and amino acid is considered to have affinities for both coenzyme-binding site and substrate-binding site of apoprotein.

2. Materials and methods

Of three Sepharose-bound derivatives of pyridoxal-P [2], we employed the 3-O-immobilized one (PLP-Seph.) as the most suitable adsorbent for the affinity chromatography of apo-tryptophanase. The preparation of PLP-Seph. was performed as reported previously [2] with a slight modification: The suspension of bromoacetamidohexyl-Sepharose [5] in 0.1 M KPB (pH 6.5) containing 0.01 M pyridoxal-P was allowed to stand for about 72 hr at 4°C in the dark. The coupling reaction product was thoroughly washed with 0.1 M KPB (pH 9.0 and pH 5.5, alternatively) and then treated with excess ethanolamine (0.05 M, pH 7.0) for 6 hr to block the remaining reactive groups of the Sepharose derivative. The effectiveness of 3-O-immobilized pyridoxine-P (PNP-Seph.) as an affinity adsorbant was also tested.

* To whom correspondence and reprint requests should be addressed.

** *Abbreviations used*: PLP or pyridoxal-P, pyridoxal 5'-phosphate; PNP, pyridoxine 5'-phosphate; pyridoxamine-P, pyridoxamine 5'-phosphate; KPB, potassium phosphate buffer.



Scheme 1. Preparation procedures of Sepharose-bound derivatives of pyridoxal 5'-phosphate, pyridoxine 5'-phosphate and pyridoxal 5'-phosphate–tryptophan complex.

This derivative was prepared by treating PLP-Seph. (200 mg, wet) with 1 mg $NaBH_4$ in 2 ml of 0.1 M KPB (pH 8.5) as described in our previous paper [2]. The preparation of Sepharose-bound pyridoxal-P-tryptophan complex (Trp-PLP-Seph.) was carried out as reported before [2]: (100 mg, wet) was suspended in 2 ml of 0.1 M KPB (pH 8.0) containing 25 mM tryptophan. Then, the resulting Schiff base was reduced with 1 mg of $NaBH_4$. The reaction product was thoroughly washed with 0.1 M KPB (pH 9.0 and pH 5.5, alternatively). The treatment of PLP-Seph., PNP-Seph. and Trp-PLP-Seph. with 6 N HCl for 12 hr at room temperature resulted in cleavage of the peptide bond in the side arm linking the chromophore to Sepharose matrix (scheme 1). From the spectral measurement of the substances liberated from the gels, the contents of the chromophores were calculated [2].

The crude preparation of apo-tryptophanase used in this study was obtained by treating cell-free extracts of *Escherichia coli* B/1t 7-A with DL penicillamine (1.5 mg/ml) followed by dialysis. The tryptophanase activity of the sample and fractions eluted from the column in affinity chromatography was assayed according to the method of Newton et al. [6] in the presence of added coenzyme. In the case of tyrosine phenol-lyase, crude extracts from *Escherichia coli* A-21 [7] were used as a crude enzyme sample after treated with DL-penicillamine. The enzyme activity was measured according to the method of Yamada et al. [7]. Protein was assayed by the procedure of Lowry et al. [8].

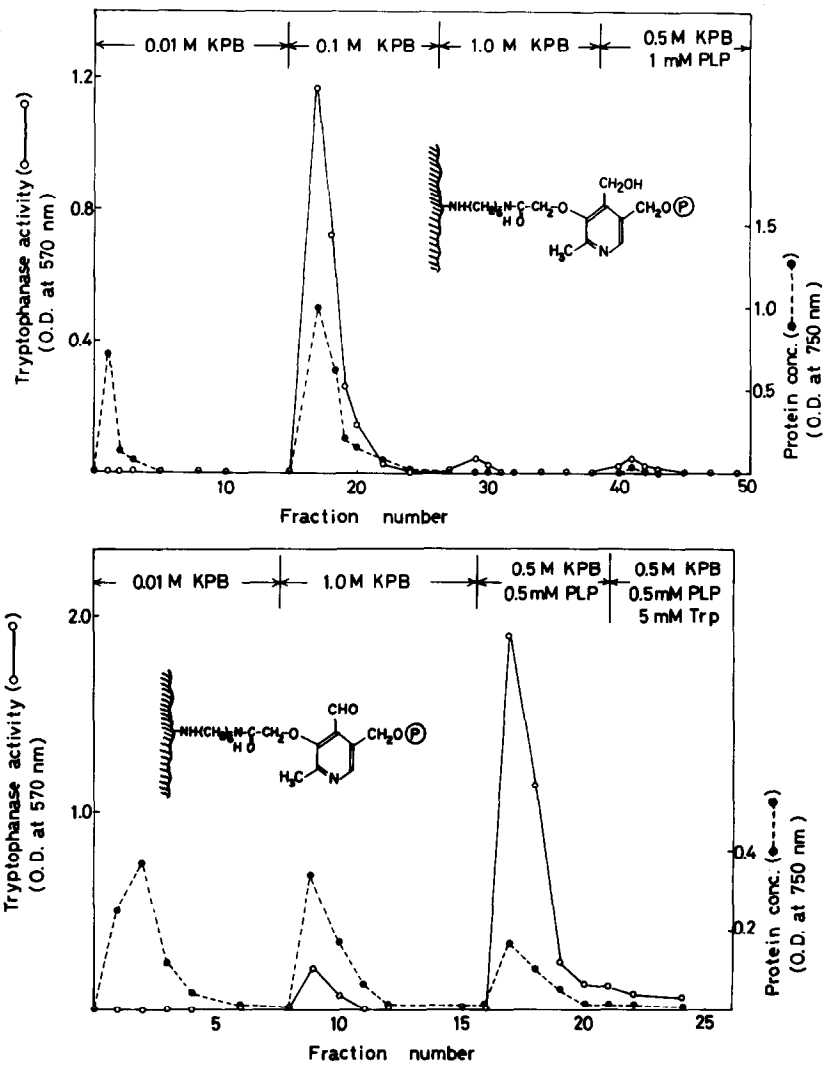


Fig.1. Elution patterns of apo-tryptophanase from Sepharose-bound pyridoxine 5′-phosphate (upper) and Sepharose-bound pyridoxal 5′-phosphate columns. Crude extracts from *Escherichia coli* B/1t 7-A (3.0 mg; specific tryptophanase activity, 0.4 units/mg-protein) was applied to a column of the indicated adsorbent (0.5 × 5 cm). Elution was carried out with the indicated eluting agents at 25° C at a flow rate of space velocity (SV) = 5 hr^{-1}. Fractions (0.5 ml) were collected and assayed for both tryptophanase activity and protein concentration.

3. Results and Discussion

Fig.1 (lower) shows a typical elution pattern of apo-tryptophanase in the crude extracts from *E. coli* applied to a column of PLP-Seph. Predominant parts of the tryptophanase activity were eluted when the column was treated with 0.5 M KPB (pH 7.0) containing 0.5mM pyridoxal-P. The specific tryptophanase activity in the fraction increased 6 to 8 times as compared with that of the initial crude extracts, although some inactivation of the enzyme was observed during the course of affinity chromatography owing to appreciable instability of apo-tryptophanase under the experimental conditions employed. The small portion of the enzyme activity which was eluted with 1.0 M KPB (pH 7.0) would be ascribable to non-biospecific adsorption of the enzyme to the adsorbent.

On the other hand, when PNP-Seph. was used as

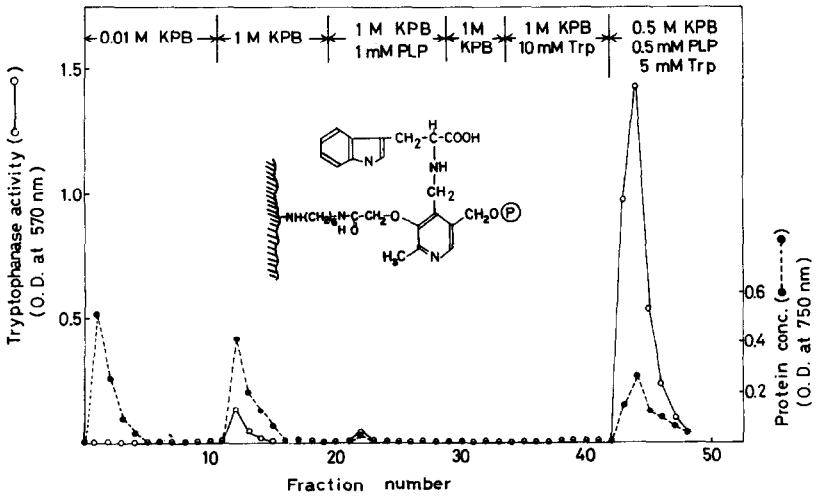


Fig.2. Elution pattern of apo-tryptophanase from Sepharose-bound tryptophan–pyridoxal 5′-phosphate complex.The experimental conditions were the same as in fig.1.

adsorbent, apo-tryptophanase was exclusively eluted with 0.1 M KPB (pH 7.0) (fig.1, upper). These results imply that the adsorption of apo-tryptophanase on PLP-Seph. would be bio-specific, whereas the adsorption on PNP-Seph. would not be bio-specific. Namely, it is concluded that the 4-formyl group of PLP-Seph. plays an important role in the bio-specific adsorption as expected readily from the well-established function of the group in the interaction between pyridoxal-P and apoprotein of vitamin B_6 enzymes.

Similarly, tyrosine phenol-lyase from *E. intermedia* was efficiently adsorbed on a column of PLP-Seph. and eluted with a mixture of 0.5 M KPB (pH 7.0) and 0.5 mM pyridoxal-P. Thus, it is demonstrated that a suitable immobilized derivative of pyridoxal-P such as PLP-Seph. serves as an excellent affinity adsorbent for different kinds of vitamin B_6 enzymes having an appropriate affinity for the coenzyme. Although pyridoxamine 5′-phosphate bound to Sepharose through its 4-aminomethyl group has been reported to be successfully used for the purification of tyrosine aminotransferase [9] and aspartate aminotransferase [10], this compound lacks a free 4-formyl group essential for the biospecific binding to most of vitamin B_6 enzymes. Hence, this immobilized derivative of Pyridoxamine-P would have only limited usefulness in the affinity chromatography of vitamin B_6 enzymes.

As shown in fig.2, apo-tryptophanase showed a more peculair and interesting behaviour toward Trp-PLP-Seph. The enzyme was desorbed only when the column was treated with a mixture of KPB, pyridoxal-P and tryptophan. Thus, Trp-PLP-Seph. was observed to be a much more specific adsorbent for apo-tryptophanase than PLP-Seph. Phosphopyridoxylamina acids, prepared by reduction of the Schiff bases formed between pyridoxal-P and various amino acids, have structures analogous to those proposed for intermediate coenzyme-substrate complexes during the course of vitamin B_6-dependent enzyme reactions. Such pyridoxylamino acids have been shown to bind efficiently to apoproteins of various vitamin B_6 enzymes, e.g., tryptophanase [11], aspartate aminotransferase [12] and tyrosine aminotransferase [13]. The immobilized derivative of the complex between pyridoxal-P and an appropriate amino acid would be of great interest and importance for the affinity chromatography of the vitamin B_6 enzyme(s) mediating the metabolism of the amino acid concerned.

Acknowledgement

The authors express their deep gratitude to Professors H. Wada, Osaka University School of Medicine, Y. Morino, Kumamoto University School of Medicine and H. Yamada, Kyoto University, Research Institute for Food Sciences for kindly providing the bacterial strains and enzyme preparation used in this study.

References

[1] Ikeda, S. and Fukui, S. (1973) Biochem. Biophy. Res. Commun. 52, 482–488.
[2] Ikeda, S., Hara, H. and Fukui, S. (1974) Biochim. Biophys. Acta 372, 400–406.
[3] Fukui, S., Ikeda, S., Fujimura, M., Yamada, H. and Kumagai, H. (1975) Eur. J. Appl. Microbiol. 1, 25–39.
[4] Fukui, S., Ikeda, S., Fuijmura, M., Yamada, H. and Kumagai, H. (1975) Eur. J. Biochem. 51, 155–164.
[5] Cuatrecasas, P. (1970) J. Biol. Chem. 245, 3059–3065.
[6] Newton, W. A., Morino, Y. and Snell, E. E. (1965) J. Biol. Chem. 240, 1211–1218.
[7] Yamada, H., Kumagai, H., Kashima, N., Torii, H., Enei, H. and Okumura, S. (1972) Biochem. Biophys, Res. Commun. 46, 370–374.
[8] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[9] Miller, J. V., Cuatrecasas, P. and Thompson, E. B. (1972) Biochim. Biophys. Acta 276, 407–415.
[10] Ryan, E. and Fottrell, P. F. (1972) FEBS Lett. 23, 73–76.
[11] Morino, Y. and Snell, E. E. (1967) J. Biol. Chem. 242, 5591– 5601.
[12] Severin, E. S., Gulyaev, N. N., Khurs, E. N. and Khomutov, R. M. (1969) Biochem. Biophys. Res. Commun. 35, 318–323.
[13] Voltattorni, C. B., Orlacchio, A., Giartosio, A., Conti, F. and Turano, C. (1975) Eur. J. Biochem. 53, 151–160.