INTERACTIONS BETWEEN AN UNUSUAL ASPARTATE AMINOTRANSFERASE FROM *RHIZOBIUM JAPONICUM* AND PYRIDOXAL-5'-PHOSPHATE STUDIED BY AFFINITY CHROMATOGRAPHY

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Received 27 March 1972

1. Introduction

Aspartate aminotransferase (AAT)(L-aspartate; 2-oxoglutarate aminotransferase, EC 2.6.1.1) from plant and mammalian sources are often resolved i.e. separated from their cofactor, pyridoxal-5'-phosphate (PLP) with some difficulty [1, 2]. In contrast, AAT from some Rhizobium bacteria is very easily resolved [3] and, as shown in the present paper, is therefore amenable to purification by affinity chromatography using PLP as ligand. This report describes interactions between PLP and AAT from *Rhizobium japonicum*, studied by an affinity chromatography system which is easier to prepare than the one recently described by Collier and Kohlhaw [4]. The results demonstrate the effectiveness of affinity chromatography both in the purification of apo-transaminases and in the study of coenzyme–apoenzyme interactions.

2. Experimental

2.1. *Bacteria*

Rhizobium japonicum, strain 392, was grown in a defined medium based on one described by Evans and Lowe [5] but with NH_4Cl and 2-oxoglutarate included instead of KNO_3 [6]. Cell free extracts were prepared as before [7].

2.2. *Enzyme assay*

AAT was estimated spectrophotometrically at 340 nm by measuring the formation of oxaloacetate with malate dehydrogenase and NADH at 30° in a total volume of 3 ml; the final concentration of reactants in the mixture were as follows: 200 mM aspartate, 20 mM 2-oxoglutarate, 5 mM NADH, 0.1 mM PLP, 50 mM potassium phosphate buffer, pH 8.0. Specific activity was expressed as μmoles of oxaloacetate formed per min per mg protein. Protein was measured with a modified biuret reagent [8].

3. Results and discussion

3.1. *Affinity chromatography: preparation of Sepharose-B-PLP derivative*

Collier and Kohlhaw [4] described a procedure for affinity chromatography of apoAAT from pig heart on a column of insolubilised PLP. They first reacted PLP with either 1,6-diaminohexane or 1,12-diaminododecane and removed excess diamine by continuous liquid–liquid extraction with ether for 24 hr. The purified N'-alkyl derivative was then coupled to Sepharose 4B. There are two disadvantages in this method, one being the possibility that the N'-alkyl derivative of PLP would be modified by activated Sepharose 4B and the other that the liquid–liquid extraction step would almost certainly need modification for the preparation of other insolubilised PLP-derivatives. We therefore devised a simpler alternative

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Abbreviations:
aspartate aminotransferase = AAT;
pyridoxal-5'-phosphate = PLP;
pyridoxamine-5'-phosphate = PMP.

method where these aforementioned disadvantages are eliminated. In the present method, 1,6-diaminohexane was first coupled to Sepharose 4B and PLP was later attached to this 'spacer' group, as follows (see fig. 1) – 50 ml of washed Sepharose 4B (Pharmacia) was suspended in 50 ml of distilled water and coupled to 1,6-diaminohexane as described by Cautrecasas [9]. After unreacted ligand was thoroughly washed off, solid PLP (0.1C g) was added to an aqueous suspension of the substituted Sepharose 4B and gently stirred for 30 min at 20°. The resulting Schiff's base was reduced by the addition of sodium borohydride at 0° to reduce foaming. It was necessary to perform the cofactor coupling cycle (i.e. PLP addition and $NaBH_4$ reduction) three times to fully saturate the free amino-groups of the Sepharose–aminohexane. The Sepharose was thoroughly washed with H_2O between cycles.

This procedure gave an insolubilised derivative of PLP where all substituents of the cofactor are available for apo-protein binding (see [10]) with the exception of the aldehyde group.

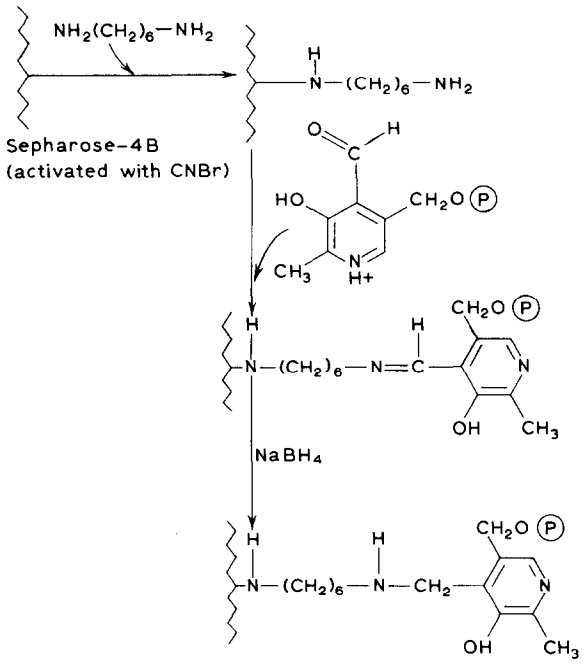

Fig. 1. Preparation of Sepharose-4B-PLP derivative. For experimental details see the text.

3.2. *Preparation of ApoAAT*

AAT from crude extracts of *R. japonicum* was resolved 100% from its coenzyme (i.e. no activity unless PLP or PMP was added) by gel-filtration on Sephadex G-200 with 0.1 M NaCl as eluant [7]. This resolution was not affected when aspartate (10 mM) or glutamate (10 mM) were added to the chloride eluant. However, AAT in crude bacterial extracts was not resolved at all when 2-oxoglutarate (10 mM) was added to the chloride eluant. AAT in crude extracts of guinea-pig or pig heart was not resolved on Sephadex G-200 when eluted with 0.1 M NaCl alone or with any of the aforementioned substrates included in the eluant.

Alternatively, AAT from crude extracts of *R. japonicum* was resolved by passage through a column of Sepharose 4B substituted with 1,6-diaminohexane and eluted with 10 mM potassium phosphate buffer pH 7.5. In this instance the cofactor was presumably removed by the formation of an imine linkage with the free amino group of the insolubilised aminohexane. Resolution of AAT was prevented on these occasions by the addition of 10 mM glutamate, aspartate, or 2-oxoglutarate to the elution buffer.

3.3. *Purification of ApoAAT*

ApoAAT from *R. japonicum*, partially purified by heat, $(NH_4)_2SO_4$ and gel filtration steps [7], was dialysed against 5 mM phosphate buffer pH 5.5 for 16 hr and then applied to a column (15 × 1.5 cm) of Sepharose 4B-PLP previously equilibrated with the same phosphate buffer. An initial protein peak was eluted with this buffer and elution was continued until no further protein was detected in the eluate. AAT apo-enzyme was then eluted with 100 mM potassium buffer pH 5.5 (fig. 2). AAT was also eluted by passing an aqueous solution of PLP (1 mg/ml) through the column. This purification procedure, the rationale for which is explained in sect. 3.4, gave a 170-fold purification of the enzyme with 69% recovery.

3.4. *Binding of ApoAAT to Sepharose-4B-PLP*

The binding of apoAAT from *R. Japonicum* to Sepharose 4B-PLP was due to specific apoenzyme–coenzyme interactions and not to nonspecific ionic reactions. This was based on the following evidence: (a) apoAAT was not bound or retarded by Sepharose 4B to which PLP was not attached, (b) holoAAT from

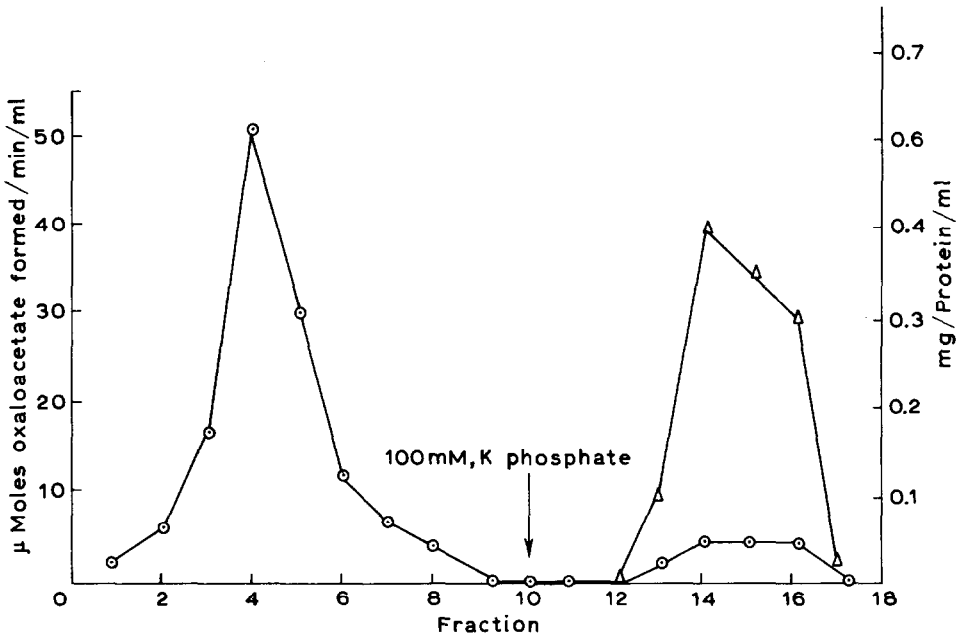

Fig. 2. Affinity chromatography of apoAAT on Sepharose-4B-PLP. For experimental details see the text. (o—o—o): A_{280}; (△–△–△): apoAAT.

pig heart was not bound or retarded on the Sepharose 4B-PLP column, (c) apoAAT was not bound to a column of Sepharose 4B-pyridoxal, prepared as outlined in sect. 3.1, (d) apoAAT bound to Sepharose-4B-PLP could be eluted with free PLP or by increasing the phosphate concentration in the elution buffer; as shown below, phosphate competes with apoAAT for the PLP binding site and (e) the binding or elution of apoAAT was not affected by 0.1 M NaCl.

Maximum binding of apoAAT from *R. japonicum* to the Sepharose-PLP column occurred when elution was performed with either (1) less than 10 mM potassium phosphate pH 5.5 or (2) 20 mM aspartate or glutamate and less than 10 mM potassium phosphate pH 7.5. Addition of 10 mM aspartate, glutamate or 2-oxoglutarate to the phosphate eluant at pH 5.5 did not affect the binding of the apoenzyme; however when 2-oxoglutarate was added instead of aspartate or glutamate to phosphate elution buffer at pH 7.5, the apoenzyme was not bound on the column.

The binding of apoAAT to Sepharose-PLP and the recovery of AAT activity after reactivation of apoenzyme with PLP (or PMP) were both strongly inhibited by phosphate; the K_i for phosphate was 2×10^{-3} M. This is probably due to competition between the 5′-phosphate group of PLP and free phosphate monoanion for the same site on the apoenzyme [11] and indicates that the 5-phosphate group of insolubilised cofactor is mainly responsible for binding the apoenzyme. Although we have no explanation at present for the influence of acidic pH or of amino acids at neutral pH on the binding of apoAAT to insolubilised PLP it could be due to conformational changes which are induced in the protein under these conditions and which are necessary for efficient combination with PLP.

Acknowledgements

We thank the Agricultural Institute (An Foras Taluntais) and the Irish Committee of the International Biological Programme for financial support. E.R. thanks the Department of Education, Dublin for a research scholarship.

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