

The Purification of Nicotinamide Nucleotide-Dependent Dehydrogenases on Immobilized Cofactors

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1. The general applicability of affinity chromatography to the purification of nicotinamide nucleotide-dependent dehydrogenases on immobilized cofactors is illustrated with several examples taken from crude systems. 2. Methods for overcoming the inevitable loss of selectivity experienced with these polymers are suggested. Effective use of the appropriate nucleotide, the second substrate and other interacting ligands can be made to selectively alter the chromatographic behaviour of the desired enzyme.

The exploitation of biological specificity as a basis for enzyme purification is now an established procedure (Cuatrecasas & Anfinsen, 1971*a,b*). Early efforts were concentrated on adsorbents specific for individual macromolecules (Cuatrecasas *et al.*, 1968), and recent developments have led to the introduction of polymers selective for groups of macromolecules (Arsenis & McCormick, 1966; Collier & Kohlhaw, 1971; Kauffman & Pierce, 1971). Likewise the application of immobilized nucleotides as adsorbents for affinity chromatography has further extended the usefulness of the technique (Lowe & Dean, 1971). Enzyme characteristics have been utilized as a basis to effect good resolution of some complex mixtures (Lowe *et al.*, 1972).

The general applicability of these adsorbents has been qualified by O'Carra & Barry (1972) who claimed that some loss of specificity would be experienced. In the present paper we describe how immobilized nicotinamide nucleotides can be successfully employed for the purification and separation of several nicotinamide nucleotide-dependent dehydrogenases from crude systems. Methods for increasing the selectivity and hence the versatility of this approach are suggested.

Materials and Methods

Materials

CNBr and ϵ -aminohexanoic acid were obtained from R. N. Emanuel Ltd., Wembley, Middx., U.K. CM-cellulose, nicotinamide- and adenine-nucleotides were obtained from Whatman Biochemicals, Maidstone, Kent, U.K. and Sepharose 4B was purchased from Pharmacia (G.B.) Ltd., London W.5, U.K. L-Threonine and all other chemicals were of the highest purity available from BDH Chemicals Ltd., Poole, Dorset, U.K.

Methods

ϵ -Aminohexanoic acid was coupled to Sepharose 4B by the CNBr activation technique of Axen *et al.* (1967). The immobilized nucleotide derivatives were prepared by coupling the appropriate nucleotide to ϵ -aminohexanoyl-Sepharose by the carbodi-imide reaction exactly as described by Larsson & Mosbach (1971). Chromatographic analyses to determine binding (β) were performed as described by Lowe & Dean (1971).

Except where stated, columns were 5 mm \times 20 mm of the appropriate immobilized nucleotide derivative. The columns were equilibrated by washing with at least 10 volumes of 10 mM-KH₂PO₄-KOH buffer, pH 7.5. Enzyme samples (50 μ l) were applied to the top of the moist column and allowed to run into the bed. The enzyme was washed into the column with a small volume of the equilibration buffer. The column was then washed with the same buffer (at least 6 ml) to remove non-adsorbed protein before application of a linear gradient of KCl (0-1 M) in the same buffer. Fractions (1.6 ml) were collected and assayed for protein, KCl (conductivity) and enzyme activity. Flow rates were maintained between 8 and 10 ml/h.

Pulse elution was performed as follows: nucleotide or salt 'pulses' in appropriate buffers (200 μ l) were applied by the same method as described above for the enzyme sample application.

The *Pseudomonas oxalaticus* extract was prepared by the method of Blackmore & Turner (1971) and the extract from *Rhodospseudomonas spheroides* by the method of Bergmeyer *et al.* (1967). The yeast extract was prepared by the method described by Easterby & Rosemeyer (1972). All extracts were dialysed against the equilibration buffer before chromatography.

Enzymes were assayed according to the methods cited by Barman (1969) and protein by the ultra-violet absorption method of Warburg & Christian

(1931) or the biuret methods of Gornall *et al.* (1949) and Itzhaki & Gill (1964).

Results and Discussion

Selection of nucleotide

The selection of the appropriate nucleotide for the particular task in hand is a consideration of prime importance for the successful application of affinity chromatography on immobilized cofactors. Thus for NAD-dependent dehydrogenases a matrix containing covalently attached NAD⁺ yielded satisfactory results, but when the adsorbent comprised immobilized NADP⁺ these enzymes could be eluted by the equilibration buffer, that is before any increase was made in the ionic strength. Table 1 demonstrates how the behaviour of *Ps. oxalaticus* L-threonine dehydrogenase was dependent on the nature of the immobilized nucleotide. Good resolution of the enzyme was achieved when the polymeric support contained covalently bound NAD⁺, NADH, ADP or 5'-AMP but little or no purification was obtained with adsorbents containing NADP⁺, NADPH, 2'-AMP or 3'-AMP. In all cases, the bulk of the protein was eluted before 150 mM-KCl. This is in accord with the behaviour observed with other NAD-dependent

dehydrogenases (Lowe *et al.*, 1972; Lowe & Dean, 1973). Fig. 1 shows that D-3-hydroxybutyrate dehydrogenase from a crude extract of *Rh. spheroides* is bound to ϵ -aminohexanoyl-NAD⁺-Sephacrose.

Table 1. Binding of *Ps. oxalaticus* L-threonine dehydrogenase to immobilized nucleotides

'Binding' refers to a measure of the strength of the enzyme-immobilized cofactor interaction and is the concentration (mM) of KCl at the centre of the enzyme peak when the enzyme was eluted with a linear gradient of KCl. Chromatographic analyses were performed as described by Lowe & Dean (1971) and Lowe *et al.* (1972).

Immobilized nucleotide	Binding
NAD ⁺	380
NADH	380
ADP	500
5'-AMP	270
NADP ⁺	0
NADPH	100
2'-AMP	150
3'-AMP	70

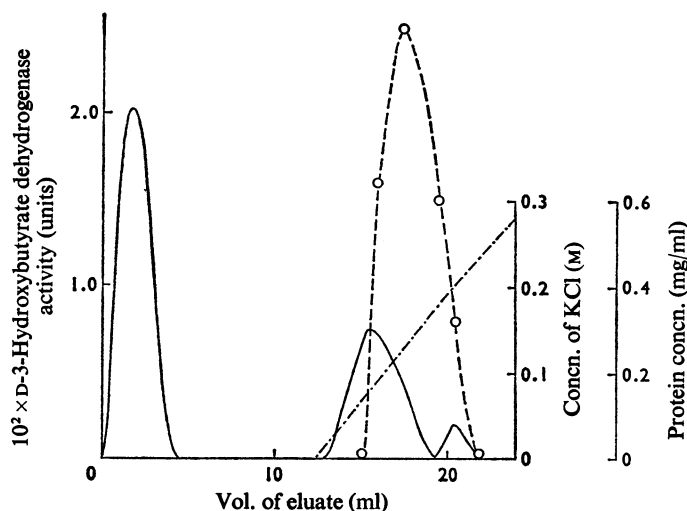


Fig. 1. Affinity chromatography of D-3-hydroxybutyrate dehydrogenase from a crude extract of *Rh. spheroides* on ϵ -aminohexanoyl-NAD⁺-Sephacrose

A sample (100 μ l) of the dialysed bacterial extract was applied to a 5 mm \times 20 mm column of ϵ -aminohexanoyl-NAD⁺-Sephacrose equilibrated with 0.05 M-Tris-HCl buffer, pH 8.0. Non-adsorbed protein was washed off with 10 ml of the same buffer and the enzyme eluted with a KCl gradient (0–0.5 M; 20 ml total volume) in 0.05 M-Tris-HCl buffer, pH 8.0. Fractions (1.6 ml) were collected at a flow rate of 8 ml/h and D-3-hydroxybutyrate dehydrogenase activity (○) assayed in the effluent. Protein concentration (—) and KCl concentration (---) were also measured.

Elution with a salt gradient yielded enzyme with increased specific activity. In contrast, no increase in specific activity was obtained by chromatography of the crude bacterial extract on ϵ -aminohexanoyl-NADP⁺-Sephacrose, as the enzyme was eluted in the void volume.

NADP-linked dehydrogenases have been purified on matrices containing either NAD⁺ or NADP⁺ although, in general, polymers containing covalently attached NADP⁺ yielded more satisfactory results. Thus D-glucose 6-phosphate dehydrogenase could be enriched 30-fold from a crude extract of *Saccharomyces cerevisiae* by chromatography on ϵ -aminohexanoyl-NADP⁺-Sephacrose, but it could only be purified 6-fold on an equivalent polymer containing immobilized NAD⁺. The enzyme was more strongly retained on the NADP⁺-linked polymer and parallels the results obtained with commercially purified enzymes (Lowe *et al.*, 1972).

All three enzymes in the systems under investigation were eluted in the void volume when the extracts were chromatographed on a control polymer comprising ϵ -aminohexanoyl-Sephacrose to which no nucleotide had been attached.

Techniques for improving selectivity

Selection of the appropriate nucleotide provides a basis for the gross separation of NAD- from NADP-dependent dehydrogenases. Additional resolution of adsorbed enzymes can be achieved through development of the selected polymer with an ionic gradient. Alternative eluent conditions can be exploited to accomplish improved resolution of specific enzymes. In the following examples 'pulse' or stepwise elution with the complementary nucleotide facilitated the release of dehydrogenases from immobilized nucleotide columns (Table 2). A 200 μ l 'pulse' of 20 mM-NAD⁺ eluted D-3-hydroxybutyrate dehydrogenase from ϵ -aminohexanoyl-NAD⁺-Sephacrose with enhanced specific activity, whereas an equivalent 'pulse' of NADP⁺ or D-3-hydroxybutyrate failed to effect elution. Similarly, a 200 μ l 'pulse' of 50 mM-NAD⁺ eluted adsorbed *Ps. oxalaticus* L-threonine dehydrogenase from immobilized NAD⁺. In a parallel experiment a 'pulse' of NADP⁺ eluted D-glucose 6-phosphate dehydrogenase from ϵ -aminohexanoyl-NADP⁺-Sephacrose.

As noted by Lowe & Dean (1973) often the reduced form of the coenzyme is a more effective eluent than the oxidized form; a 200 μ l 'pulse' of 5 mM-NADH was sufficient to elute *Ps. oxalaticus* L-threonine dehydrogenase from ϵ -aminohexanoyl-NAD⁺-Sephacrose. With systems comprising relatively pure enzymes, 'pulse' elution can yield quantitative recoveries (Mosbach *et al.*, 1972; Lowe *et al.*, 1972; Lowe & Dean, 1973), whereas with crude systems, recoveries of enzyme activity can be unacceptably

low (Table 2a). As shown in Table 2(b), additional 'pulses' of nucleotide effect a further release of the enzyme and quantitative recoveries of enzyme activity can be achieved by 'pulsing' with 500 mM-KCl. It is suggested that the low recoveries observed with single 'pulses' were because there was insufficient nucleotide to effect elution, or as a result of cofactor binding by other nicotinamide nucleotide-dependent enzymes.

A linear gradient of the appropriate nucleotide was a considerably more effective technique for the selective elution of enzymes from columns of immobilized cofactors, but the expense of these cofactors limits the general applicability of the procedure. Fig. 2(a) shows how L-threonine dehydrogenase from a crude extract of *Ps. oxalaticus* can be quantitatively eluted from ϵ -aminohexanoyl-NAD⁺-Sephacrose by a linear gradient of NAD⁺ (0–50 mM). The peak of enzyme activity was eluted at 27 mM-NAD⁺.

The selectivity of group-specific adsorbents can also be enhanced by exploiting the nature of the reaction catalysed by the complementary enzymes. Most nicotinamide nucleotide-dependent dehydrogenases are thought to have ordered kinetic mechanisms in which the nicotinamide nucleotide compulsorily binds first, before formation of the ternary complex with the second substrate can occur. It is conceivable, therefore, that the presence of the second substrate will selectively alter the chromatographic behaviour of the required enzyme on adsorbents containing covalently attached nicotinamide nucleotides. Figs. 3(a) and (b) illustrate the effect of the second substrate, L-threonine, on the chromatographic behaviour of L-threonine dehydrogenase on ϵ -aminohexanoyl-NAD⁺-Sephacrose when eluted with a linear gradient of salt. Binding of the enzyme was increased in the presence of 10 mM-L-threonine and the resolution from the inert protein was improved with a concomitant increase in specific activity of the enzyme. The behaviour of L-threonine dehydrogenase was influenced by the presence of L-threonine when the column was developed with a linear NAD⁺ gradient; binding was decreased when the eluent was supplemented with 10 mM-L-threonine (Fig. 2). Elution of the enzyme could not be effected with a linear gradient of threonine (0–0.1 M) alone.

The apparent difference in the effect of L-threonine on the elution of L-threonine dehydrogenase by ionic and nucleotide gradients can be reconciled on the following basis. In both cases the formation of a ternary complex between the enzyme, immobilized NAD⁺ and threonine was promoted. The increased binding observed when the column was developed in a KCl gradient reflects the greater stability of the ternary complex compared with the binary complex in the absence of threonine. When the ternary complex was subjected to a linear NAD⁺ gradient

Table 2. 'Pulse' elution of dehydrogenases from matrices containing covalently attached nicotinamide nucleotides

Details of the 'pulse' elution procedure are given in 'methods'. Chromatographic conditions are described in the legends of the appropriate figures. D-3-Hydroxybutyrate dehydrogenase (0.5 unit) and L-threonine dehydrogenase (2.2 units) were applied to the respective columns.

Enzyme	Source	Nucleotide immobilized on matrix	Eluent nucleotide	Concn. (mM)	Recovery of enzyme activity (%)	Accumulated recovery of enzyme activity (%)
(a) Single 'pulse' experiments						
D-3-Hydroxybutyrate dehydrogenase	<i>Rh. spheroides</i>	NAD ⁺	NAD ⁺	20	55	55
			NADP ⁺	20	0	0
L-Threonine dehydrogenase	<i>Ps. oxalaticus</i>	NAD ⁺	NAD ⁺	50	25	25
			NADH	5	40	40
(b) Sequential 'pulse' experiments						
L-Threonine dehydrogenase	<i>Ps. oxalaticus</i>	*NAD ⁺	†(1) NADH	5	25	25
			(2) NADH	5	25	50
			(3) NAD ⁺	50	0	50
			(4) KCl	500	68	118
		†NAD ⁺	(1) NADH	5	59	59
			(2) NADH	5	36	95
		†NAD ⁺	(1) NAD ⁺	50	36	36
			(2) NAD ⁺	50	36	72
			(3) NADH	5	18	90

* Equilibration buffer, 10mM-KH₂PO₄-KOH, pH7.5.

† Equilibration buffer, 10mM-KH₂PO₄-KOH, pH7.5 containing 100mM-KCl.

‡ Sequence of 'pulses' applied (200 μl).

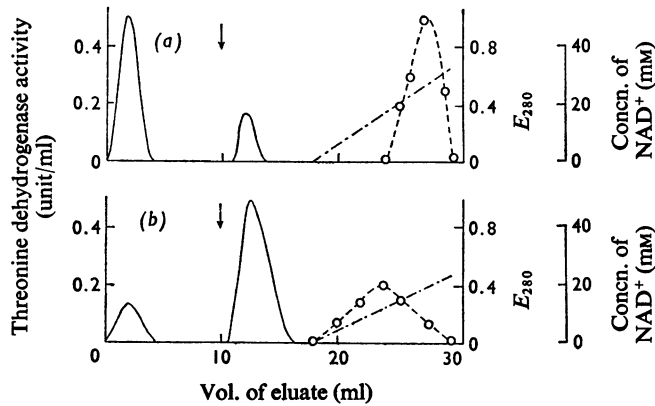


Fig. 2. Effect of L-threonine on the binding of L-threonine dehydrogenase to ϵ -aminohexanoyl-NAD⁺-Sepharose

A sample (100 μ l) of the crude extract was applied to a 5 mm \times 20 mm column of the gel equilibrated with 10 mM-potassium phosphate buffer, pH 7.5. Non-adsorbed protein was washed off with 10 ml of the same buffer and inert protein was eluted by increasing the KCl concentration to 100 mM (arrow). L-Threonine dehydrogenase was eluted by a linear gradient of NAD⁺ (0–50 mM; 20 ml total volume) in 10 mM-phosphate buffer, pH 7.5 containing 100 mM-KCl. Enzyme (\circ), inert protein (—) and NAD⁺ concentration (---) were assayed in the effluent. In (b) conditions were as in (a) except that 10 mM-L-threonine was included in all buffers.

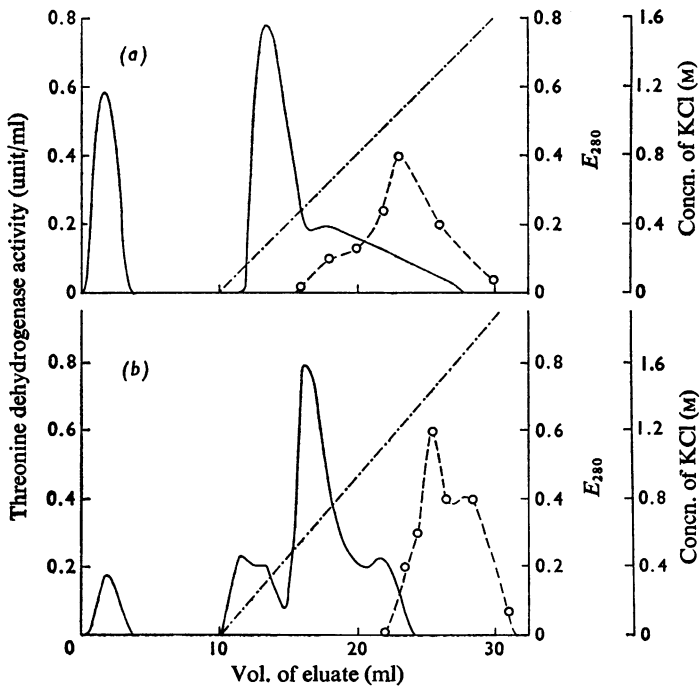


Fig. 3. Effect of L-threonine on the binding of L-threonine dehydrogenase to ϵ -aminohexanoyl-NAD⁺-Sepharose

Chromatographic analyses were performed as described for Fig. 2 except that the columns were developed with a linear KCl gradient (0–1.0 M; 20 ml total volume) in 10 mM-potassium phosphate buffer, pH 7.5. L-Threonine dehydrogenase activity (\circ), protein (—) and KCl concentration (---) were assayed in the effluent. (a) No threonine added; (b) plus threonine (10 mM) in all buffers.

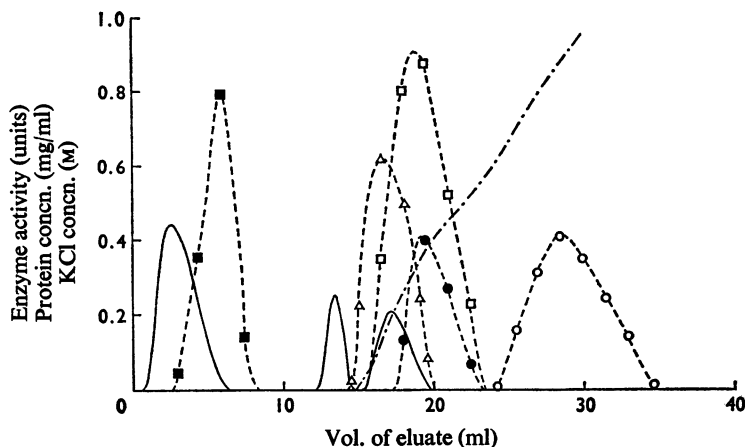


Fig. 4. Affinity chromatography of a crude yeast extract on ϵ -aminohexanoyl-NADP⁺-Sephrose

A sample (100 μ l) of a dialysed yeast extract was applied to a 5 mm \times 20 mm column of ϵ -aminohexanoyl-NADP⁺-Sephrose equilibrated with 10 mM-potassium phosphate buffer, pH 7.5. Non-adsorbed protein was washed off with 10 ml of the same buffer and the column was developed with a KCl gradient (0–1.0 M; 20 ml total volume) in 10 mM-potassium phosphate buffer, pH 7.5. D-Glucose 6-phosphate dehydrogenase (○), L-malate dehydrogenase (△), EC 1.1.1.37, glutathione reductase (●), D-glyceraldehyde 3-phosphate dehydrogenase (■), EC 1.2.1.12, and alcohol dehydrogenase (□) were assayed in the effluent. Protein (—) and the concentration of KCl (---) were also measured.

supplemented with threonine, release of the enzyme from the matrix would be followed by formation of a tighter ternary complex in free solution. This would prevent the subsequent partition of the enzyme through the column (Lowe *et al.*, 1973).

Any ligand that interacts with the macromolecule in such a way as to alter its chromatographic behaviour can be utilized to increase the selectivity of the desorption phase. Thus, Mosbach *et al.* (1972) have shown that a linear gradient (0–0.15 M) of the competitive inhibitor, salicylate, effects an efficient separation of D-glyceraldehyde 3-phosphate dehydrogenase and lactate dehydrogenase from *N*⁶-(6-aminohexyl)-AMP-Sephrose. Other techniques based on similar principles can clearly be envisaged.

Other considerations

It is a formidable task to predict the behaviour of nicotinamide nucleotide-linked dehydrogenases when crude extracts are chromatographed on adsorbents containing covalently attached nucleotides. The following observations demonstrate some of the parameters that could influence the binding of enzymes under such conditions.

The competition of two dehydrogenases at saturating concentrations for the same immobilized nucleotide will lead to a mixture of the two enzymes being adsorbed, even though there may be considerable

differences in their apparent affinities. Fig. 4 illustrates the behaviour of several NAD(P)-dependent dehydrogenases when a crude yeast extract is chromatographed on a column of ϵ -aminohexanoyl-NADP⁺-Sephrose and developed with a linear salt gradient. Considerable purification of D-glucose 6-phosphate dehydrogenase (EC 1.1.1.49) was achieved. Glutathione reductase (EC 1.6.4.2) was strongly retained, but was not resolved from alcohol dehydrogenase (EC 1.1.1.1). Application of specific elution principles could assist the resolution of this complex mixture. Note that the conditions chosen for this experiment were not necessarily optimal to achieve the desired separations.

The presence of nucleic acid components and non-specific proteins in crude extracts could also decrease the effectiveness of these adsorbents by direct interaction with the bound nucleotides. Considerable advantage may thus be found in the introduction of a preliminary purification step (e.g. ion-exchange chromatography) before fractionation on the affinity adsorbent. A preliminary purification step before affinity chromatography has been widely used, particularly when either immobilized cofactors or prosthetic groups have been exploited (Arsenis & McCormick, 1966; Collier & Kohlhaw, 1971; Gaudie & Hillcoat, 1972; Ryan & Fottrell, 1972). Thus the application of conventional chromatographic procedures together with affinity chromato-

graphy could be adapted to obtain relatively pure enzymes from crude systems.

Although the polymers used in the present study are heterogeneous (Lowe *et al.*, 1973) they hold the distinct advantage over the defined polymer described by Mosbach *et al.* (1972) in their simplicity of preparation.

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