AFFINITY CHROMATOGRAPHY OF ENZYMES ON INSOLUBILIZED COFACTORS

C.R. LOWE and P.D.G. DEAN

Department of Biochemistry, The University, P.O. Box 147, Liverpool L69 3BX, England

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1. Introduction

The use of affinity chromatography in the purification of enzymes is increasing in importance. To date, the technique has been applied primarily to mono-substrate enzymes [1, 2]. Cuatrecasas [1, 3] has described the more important experimental conditions necessary for satisfactory binding of proteins to insolubilized ligands: thus it is suggested that the ligand must be sufficiently distant from the matrix backbone to minimize steric interference with the binding process.

This report describes the use of insolubilized cofactors as a means of resolving those groups of enzymes that utilize them in bi- or multi-substrate reactions. The NAD(P)- (that is NAD or NADP)-linked dehydrogenases provide a basis on which the usefulness of this technique can be estimated.

We have demonstrated the effectiveness of insolubilized cofactors in separating mixtures of proteins by affinity chromatography. Our work suggests that parameters such as the conformation of the bound cofactor have a more profound effect on binding* of the enzyme than the distance of the cofactor from the matrix backbone.

* 'binding' refers to a measure of the enzyme insoluble substrate–cofactor interaction which is defined as the concentration of salt required to elute a particular enzyme.

2. Materials and methods

Enzymes were purchased from Boehringer (Mannheim) except for MDH and G3PDH, which were obtained from Sigma (London) Ltd., Wembley, England. from extracts of *Pseudomonas oxalaticus* kindly supplied by Dr. J.M. Turner. The water-soluble carbodiimide was 1-cyclohexyl-3-(-2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulphonate and was supplied by R. Emmanuel Ltd., Wembley, England. Cellulose was kindly donated by Whatman Biochemicals, Maidstone, Kent.

Cofactors were coupled directly to cellulose by the cyanogen bromide technique described by Axen, Porath and Ernbäck [4] as follows: cellulose (20 g) was washed with distilled water and suspended in about 50 ml water. Cyanogen bromide (6 g) was added and the pH maintained at pH 11 ± 0.2 by addition of 8 N KOH. The reaction was carried out at 20 ± 2°. After 8 min the cellulose was washed with 2 litres of ice cold 0.1 M $Na_2CO_3/NaHCO_3$ buffer (pH 9.5) and a solution of NAD(P) (0.1 g) in the same buffer was added. The mixture was then stirred at 4° for 8 hr and unreacted NAD removed by washing with water.

Abbreviations:

GDH : L-glutamate dehydrogenase
G6PDH: D-glucose-6-phosphate dehydrogenase
ICDH : threo-D_s-isocitrate dehydrogenase
MDH : L-malate dehydrogenase
LDH : L-lactate dehydrogenase
TDH : L-threonine dehydrogenase
ADH : alcohol (liver) dehydrogenase
G3PDH: D-glyceraldehyde-3-phosphate dehydrogenase

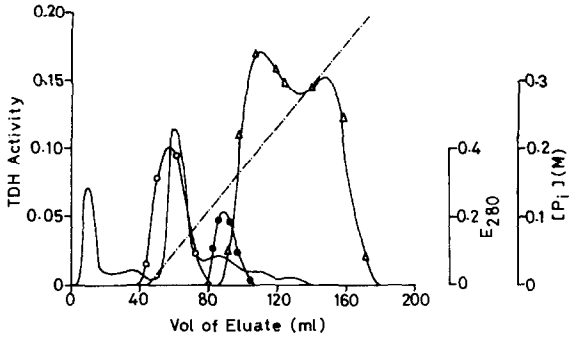

Fig. 1. Chromatography of a crude dialysed extract of *Ps. oxalaticus* on NAD-cellulose. A 0.5 ml sample was applied to a 1.4 cm × 9.0 cm column of NAD-cellulose equilibrated with 10 mM phosphate buffer, pH 7.5. Non-absorbed protein was washed off with 40 ml of the same buffer and enzyme eluted with a phosphate gradient (0.01–0.50 M, 160 ml total, at a flow rate of 30 ml/hr).
G6PDH (○), LDH (●), TDH (△) activities and protein (—) were assayed in the effluent.

The NAD-cellulose was then packed into a column. In general about 80% of the material absorbing at 260 nm was bound to the column. This was determined by difference and also by direct absorbance using a Perkin Elmer 356 double beam spectrophotomer.

ϵ-Amino eaproate, glycylglycylglycine and *o*-aminophenol were coupled to cellulose in a similar fashion. ϵ-Amino caproyl- and glycylglycylglycyl-cellulose were then coupled to NAD(P) using the method described for water-soluble carbodiimide by Cuatrecasas [1].

Diazo-NAD-cellulose was prepared by treating *o*-hydroxyanilino-cellulose (15 g) with diazotized benzidine (ex 1 g benzidine-HCl) [7] at 0° for 10 min. The cellulose was then washed with ice-cold water for 10 min and suspended in a solution of NAD (50 mg) in 0.1 M $Na_2CO_3/NaHCO_3$ buffer, pH 9.0 (15 ml). The suspension was stirred overnight at 0° and washed thoroughly with a large volume of ice-cold distilled water.

The enzymes were assayed according to the methods cited by Barman [5] and protein in the column effluents was assayed by the method of Warburg and Christian [6].

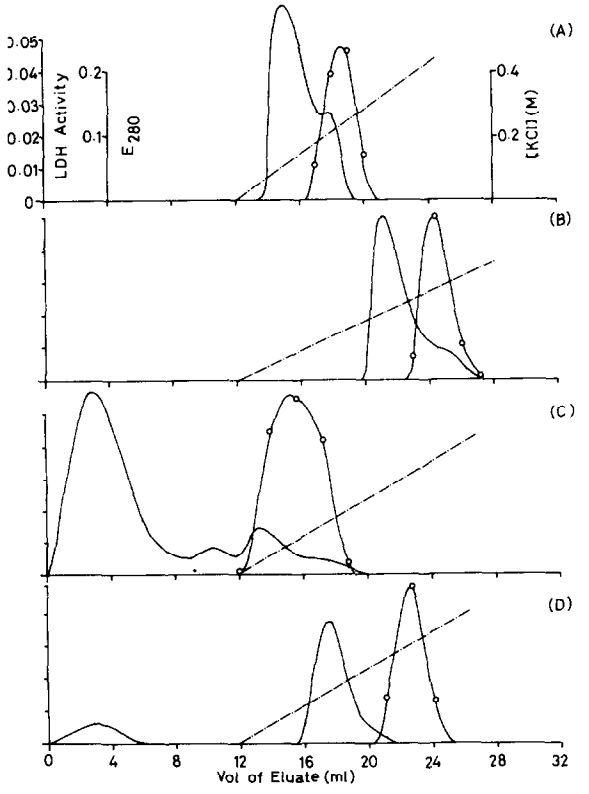

Fig. 2. Affinity chromatography of LDH–albumin mixtures on NAD-cellulose and cellulose with NAD bound through extensions of varying length. (A) NAD-cellulose, (B) ϵ-amino-caproyl-NAD-cellulose, (C) diazo-NAD-cellulose, (D) glycyl-glycylglycyl-NAD-cellulose. A 50 μl sample containing 1.85 units LDH and 0.8 mg BSA was applied to a column (5 mm × 20 mm) of the appropriate NAD-cellulose equilibrated with 10 mM Pi buffer, pH 7.5 Non-absorbed protein was washed off with the same buffer and the column eluted with a KCl gradient (0–0.5 M) in 10 mM Pi, pH 7.5, 20 ml total. LDH (○) and BSA (—) were assayed in the effluent.

3. Results

Fig. 1 illustrates the chromatography of a dialysed cell-free extract of *Ps. oxalaticus* on NAD-cellulose. TDH activity is resolved into two peaks which appear after application of the salt gradient. The enzyme (TDH) is bound more strongly to the NAD-cellulose than, not only the majority of the inert protein, but also the activities of LDH and G6PDH. The overall purification of the two peaks of TDH was at least 150- and 450-fold respectively.

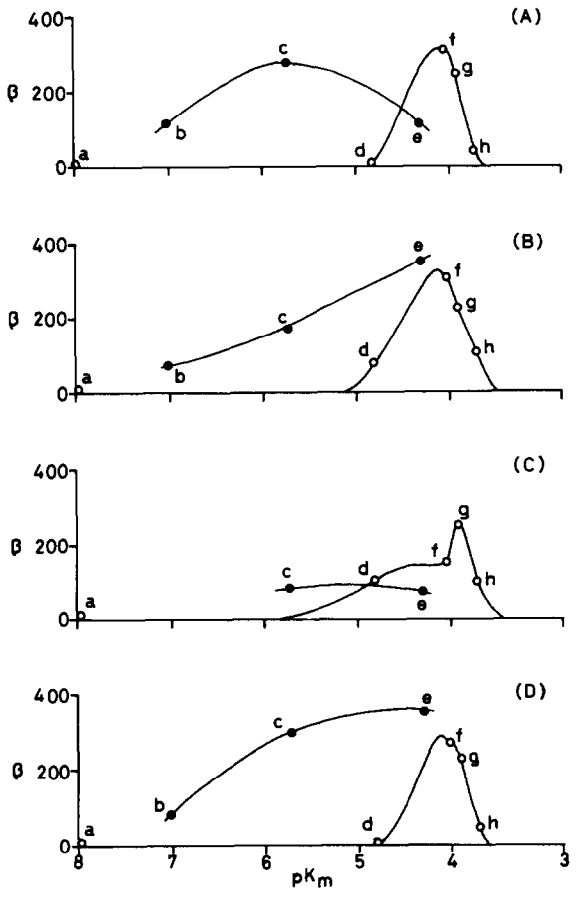

Fig. 3. The binding of NAD(P)-linked dehydrogenases to several types of NAD(P)-cellulose. (A) NAD-cellulose, (B) ε-aminocaproyl-NAD-cellulose, (C) diazo-NAD-cellulose, (D) NADP-cellulose. The binding (β) represents the concentration of KCl required to elute the enzyme and is plotted against the values for the negative logarithm of the Michaelis constant (literature values [5]). (a) G3PDH, (b) ICDH, (c) G6PDH, (d) ADH, (e) GDH, (f) LDH, (g) TDH, (h) MDH.

Fig. 2 shows the separation of LDH from serum albumin on a series of NAD-cellulose columns. Albumin binding is always weaker than LDH binding and moreover, much more susceptible to the manner in which the NAD is bound. LDH binding is remarkably unaffected by the distance of the NAD from the matrix backbone, even when the purine moiety is attached to position 8 (fig. 2 C) rather than via the NH_2 group (fig. 2 A, B and D).

The behaviour of a series of NAD(P)-linked dehydrogenases on NAD-, NADP- and extended NAD-cellulose columns produces somewhat surprising results: fig. 3 shows the relationship between the negative logarithm of the Michaelis constant (pK_m NAD(P)) for the free cofactor (as distinct from the insolubilized cofactor) and the binding of NAD(P)-linked dehydrogenases to NAD-cellulose (fig. 3 A), to two types of extended NAD-cellulose (fig. 3 B and C) and to NADP-cellulose (fig. 3 D).

Several interesting points emerge: first, not only do most NADP-linked dehydrogenases bind to NAD-cellulose, but most NAD-linked enzymes bind to NADP-cellulose. Second, the binding of NAD(P)-linked dehydrogenases to NAD-cellulose and to those in which a spacer molecule is included is not a simple function of the pK_m NAD(P)*. The binding to these columns exhibits clearly defined maxima over a narrow range of pK_m NAD(P). Third, G3PDH and ADH are not bound to NAD- or NADP-cellulose.

An attempt to illustrate the relative specificity of NAD(P)-linked dehydrogenases for their preferred co-factors is shown in fig. 4.

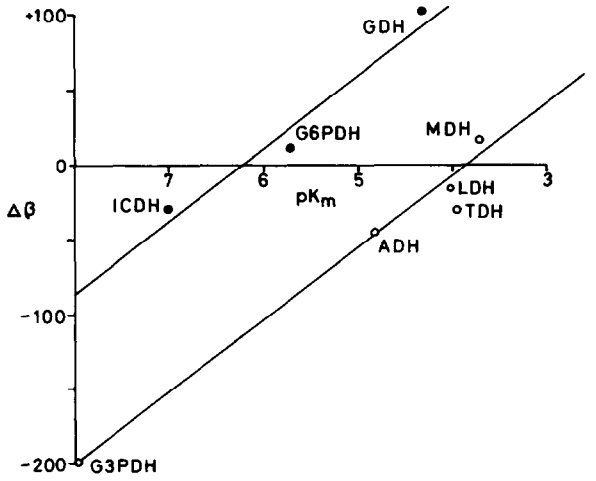

Fig. 4. The binding of NAD(P)-linked dehydrogenases to NAD(P)-cellulose. $\Delta\beta$ represents the difference in binding onto NAD- and NADP-cellulose expressed as a percentage of the mean, i.e.

$$\Delta\beta = \frac{\beta\text{NADP} - \beta\text{NAD}}{\frac{1}{2}(\beta\text{NADP} + \beta\text{NAD})} \times 100\%.$$

* pK_m NAD(P) is taken as the value for the first mole of NAD(P) which binds to the enzyme.

4. Discussion

Preliminary studies with crude bacterial extracts (fig. 1) have shown that NAD-cellulose is an effective affinity chromatography medium for the isolation of TDH. Experiments with commercially available purified dehydrogenases show that in general they are bound more tightly to their respective preferred cofactors (fig. 4). However, quantitative conclusions about their behaviour are difficult to draw in view of many unknown parameters. Besides the steric and diffusional limitations imposed by the cellulose matrix, the cofactor columns exhibit weak ion-exchange properties. These properties arise, not only from the phosphate content of the coenzymes, but also from incomplete reaction of carboxyl groups in the carbodiimide reaction leaving uncoupled ε-amino-caproyl-cellulose and glycylglycylglycyl-cellulose. The former observation can explain why MDH, which has a relatively low affinity for unbound NAD, binds more strongly to NADP- than to NAD-cellulose (fig. 4).

Parallel studies on the interaction of phosphokinases with ATP- and ADP-cellulose (Corry, Lowe and Dean, unpublished observations) demonstrate little selective binding. This is to be expected since it is the adenine moiety of ATP that is important in its binding to kinases, and interaction with the latter is impeded in the insolubilized ATP.

The NAD(P)-linked dehydrogenases are subject to allosteric, association–dissociation, stereospecific and conformational effects, each with their associated kinetic consequences on coenzyme binding. Furthermore, although we have attempted to assay both cofactors by the E_{260}/E_{280} ratio, the existence of tightly bound cofactors to the enzymes cannot be excluded when attempting to explain these results. No relationship can be found between the behaviour of these enzymes and their stereospecificity or molecular weight.

It is suggested that the conformation of the insolubilized cofactor is an important consideration. Kaplan and Sarma [9] review the geometry of the pyridine nucleotide coenzymes with special reference to the stereospecificity of their binding to the NAD(P)-linked dehydrogenases. Covalent linkage to the adenine moiety of NAD(P) would disturb the helix transitions of the coenzyme. For most NAD(P)-linked dehydrogenases [10] except G3PDH and LDH [11], the coenzyme unfolds, and it is probably significant that these two enzymes do not bind to either NAD or NADP-cellulose (fig. 3 A and D).

These observations cast new light on the interpretation of fig. 3: decreasing pK_m NAD is associated with marked increases in the binding, except when the enzyme is known to require the folded conformation of the coenzyme. Exceptions found with diazo-coupled NAD-cellulose (fig. 3 C) columns may be explained by the inherent restrictions in the mobility of the linkage caused by the rigidity of the benzidine bridge.

This work shows that cofactors, as well as enzyme substrates, may be used for affinity chromatography, but that the generalizations outlined by other authors [1, 3, 8] may not be as significant in the case of the cofactors studied here.

Acknowledgements

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