AFFINITY CHROMATOGRAPHY OF LACTATE DEHYDROGENASE
Model Studies Demonstrating the Potential of the Technique in the Mechanistic Investigation as well as in the Purification of Multi-Substrate Enzymes

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1. Introduction

The general potential of affinity chromatography in enzyme isolation and purification is now well recognized [1–3]. No general treatment of the special problems likely to be encountered in applying it to multi-substrate enzymes seems to have been published, nor do some of the special possibilities of the technique for such enzymes seem to be generally recognized.

This paper describes model studies on lactate dehydrogenase (LDH) which explore some of these problems and possibilities. The results demonstrate that the effectiveness of affinity chromatography as a purification tool for certain types of multi-substrate enzyme may be greatly increased by taking advantage of kinetic characteristics. It is also shown that other advantages may accrue in that affinity chromatography is capable of yielding very clear-cut information about multi-substrate kinetic mechanisms. For example, the technique clearly demonstrates the compulsory–ordered mechanism of LDH and also indicates that it is the nicotinamide end of NADH which induces the binding site for pyruvate analogues, even though it is the "AMP portion" which is mainly responsible for the binding of the pyridine nucleotide to LDH [4].

2. Experimental

2.1. *Materials and methods*

The LDH 1 (H_4) used in the model experiments (fig. 3) was a crystalline preparation from pig heart supplied by Sigma Chemical Co., London, who also supplied the AMP, ADP and water-soluble carbodiimide. NADH and NAD^+ were supplied by Boehringer, Mannheim, and Sepharose 4B by Pharmacia, Uppsala. LDH was assayed essentially following the method of Fritz et al. [5], but using 0.02 M phosphate buffer, pH 7.4, instead of Tris. Protein concentrations were measured by the Lowry method [6], and the glucose internal standard was assayed by a micro-adaption of the dinitrosalicylate method [7].

2.2. *Preparation and running of the affinity column*

Sepharose 4B was substituted with aminohexyl groups (5–10 μmoles per ml of packed Sepharose) following the general procedure of Cuatrecasas [2]. The terminal amino group was then condensed with oxalate via an amide bond, to produce the insolubilized oxamate derivative, by the following procedure: To each 5 ml of packed aminohexyl-Sepharose was added 140 mg of potassium oxalate in 2 ml water, adjusted to pH 4.7, followed by the dropwise addition of 370 mg of the water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, dissolved in 1 ml water. The mixture was then stirred gently at room temp for 20 hr. At this stage the trinitrobenzene sulphonate test [2] showed that the amino groups had been completely reacted, and the Sepharose derivative was washed on a sinter-glass funnel with 1 l of distilled water.

Chromatography on this material was carried out at 15° in miniature columns (7 mm internal diameter: 4 ml bed vol) run under a 70 cm hydrostatic head of pressure. For analytical runs (e.g. fig. 3) samples were

applied in a vol of 0.4 ml. A little glucose (3–4 mg) was generally dissolved in these samples as an internal standard to provide a reliable reference point on the elution profile corresponding to "straight-through" elution. Effluent was collected as 0.4 ml fractions (i.e. 10 fractions per column vol) using a Gilson micro-fractionator. These fractions were divided into 0.05–0.2 ml aliquots for assays of LDH, glucose and protein.

When affinity chromatography was first attempted on these columns, 0.02 M phosphate, pH 6.8, was used as the irrigant. LDH was found to be quite strongly adsorbed, being eluted by an increasing salt gradient. However, a number of other proteins which were not expected to have any specific affinity for the oxamate were adsorbed and eluted in almost exactly the same way and it quickly became apparent that the adsorption was attributable to non-specific ion-exchange effects, presumably involving the ionized carboxyl group of the oxamate. An NaCl concentration of 0.5 M fully eliminated these ion-exchange effects, all the 'non-specific proteins" tested then being completely unretarded. 0.5 M NaCl in 0.02 M sodium phosphate buffer, pH 6.8, was therefore used as the routine irrigant for the columns. In view of this and other similar experiences, it seems that greater care should be exercised in testing for, and eliminating, non-specific ion-exchange effects during the development of affinity chromatography procedures.

3. Results

Lowe and Dean [3] and Mosbach et al. [8] have already described affinity chromatography of LDH and some other pyridine nucleotide-dependent dehydrogenases on columns of insolubilized NAD. One reason for choosing NAD as the insolubilized ligand is because columns of such material are likely to adsorb a wide range of NAD-dependent enzymes and thus are expected to have a general applicability in the purification of such enzymes. This, however does not seem to us to be an unqualified advantage, since what is gained in general applicability is lost in specificity.

Greater specificity might be expected from affinity chromatography on insolubilized analogues of the "specific substrates" of these dehydrogenases (e.g.

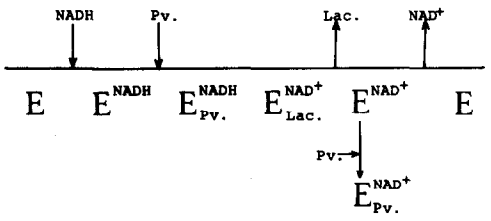

Fig. 1. Cleland-style line-diagram [10] representing currently-held views on the kinetic mechanism of LDH. (Pv. = pyruvate; Lac. = lactate).

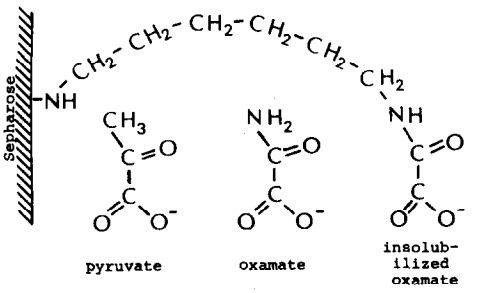

Fig. 2. Structural analogy of pyruvate, oxamate and the insolubilized, Sepharose-linked oxamate derivative.

pyruvate/lactate in the case of LDH) but a factor which may have militated against previous efforts in this direction is that many, perhaps most, pyridine nucleotide-dependent dehydrogenases are thought to have compulsory–ordered kinetic mechanisms in which the pyridine nucleotide compulsorily binds first. Such a mechsnim has been proposed for LDH (cf. [9]) and is illustrated in fig. 1. If this mechanism is correct, it is clear that straight-forward attempts at affinity chromatography on ligands kinetically analogous to pyruvate or lactate are unlikely to succeed, but an inspection of the mechanism reveals how this superficial difficulty might be turned to advantage, as demonstrated in the following paragrphs.

The most clear-cut and interesting results were obtained with the insolubilized oxamate derivative whose preparation is described above. Oxamate is an analogue of pyruvate and is an inhibitor of LDH, strictly competitive against pyruvate [11]. The structural analogy is illustrated in fig. 2 which also shows the manner in which the oxamate derivative was insolubil-

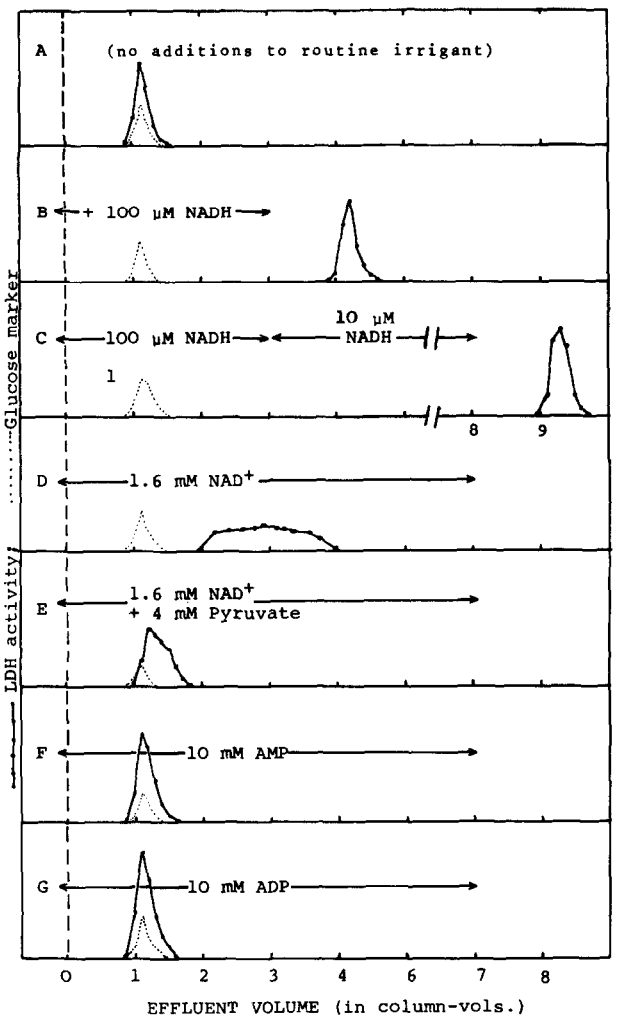

Fig. 3. Affinity chromatography of LDH 1 (H_4) from pig heart on the Sepharose-linked oxamate derivative discussed in the text. Irrigant throughout was 0.5 M NaCl in 0.02 M sodium phosphate buffer, pH 6.8, with the nucleotide additions indicated on the individual elution diagrams. The points of commencement and termination of such additions are indicated by the points of the horizontal arrows which are correlated with volume of effluent collected when such changes were made. Effluent volume was measured from time of application of sample, this zero-point in the effluent being marked with the vertical dashed line in the figure. As indicated, additions to the irrigant were commenced a little ahead of sample application. The same additions were made to the applied samples, which were made up in the routine irrigant. As mentioned in the text, a little glucose was added to all applied samples and was analysed in the effluent fractions to provide a reliable measure of "straight-through" (i.e. unretarded) volume. (AMP = adenosine 5'-monophosphate).

ized by attachment to Sepharose 4B through an "extension arm" to minimize steric hindrance of the enzyme–inhibitor interaction.

When 0.5 M NaCl was included in the irrigating buffer, no retardation of LDH on columns of the insolubilized pyruvate analogue could be detected (fig. 3 A) indicating that the enzyme had no affinity for the insolubilized inhibitor.

However, when NADH was added to the LDH samples and to the irrigating buffer, the LDH was strongly retained on the columns and remained adsorbed as long as NADH was included in the irrigant even at concentrations as low as 10 μM (fig. 3 B, C), When NADH was then omitted from the irrigant, the LDH was eluted almost exactly one column-volume later (fig. 3 B, C) just behind the tailing edge of the NADH, indicating that LDH dissociates from the column material as soon as the excess NADH is washed ahead of it.

These results are indicative of a binding-site for the pyruvate analogue which is induced in LDH on binding of NADH, but which is absent in the free enzyme, as predicted by the compulsory–ordered kinetic mechanism of fig. 1.

NADH could thus be termed a compulsory, complementary ligand, in direct contrast to the competitive counter ligands familiar in affinity chromatography.

It is clear that much more specificity is inherent in the "negative" elution achieved by discontinuation of the complementary ligand than would be achieved by "positive" elution with a counter ligand, such as pyruvate or free oxamate in the present instance, because not only does the initial presence of NADH promote the efficient separation of LDH from proteins with no affinity for the oxamate derivative but the subsequent elution of LDH by discontinuation of the NADH should leave behind any other adsorbed proteins, so long as their adsorption is not also dependent on the presence of NADH. Such other adsorbed proteins, however, would probably be eluted by a counter ligand, such as free oxamate or pyruvate.

The specificity implicit in the procedure is thus more than the equivalent of two independent affinity chromatography steps, being dependent not only on the dual affinity of LDH for NADH and for the oxamate derivative, but also on the compulsory order and interrelation of the two affinities. Additional affinity chromatography on insolubilized NAD(H) should thus be entirely superfluous.

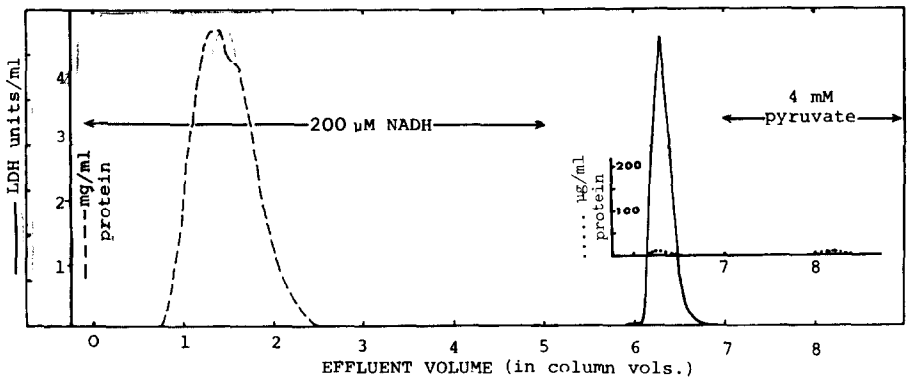

Fig. 4. Chromatographic isolation of LDH from a crude placental extract on a column of insolubilized oxamate. Human placenta, mixed with an equal vol of 0.5 M NaCl in 0.02 M phosphate buffer, pH 6.8, was homogenized (Sorval Omni Mixer, setting 8, 1 min) and centrifuged at 35,000 *g* for 30 min. The supernatant was adjusted to pH 6.8, 0.5 M NaCl and 200 μM NADH before application. Addition of NADH to irrigant was commenced shortly before application of sample. Volume of applied sample was 1.6 ml (0.4 of a column vol) but larger samples may be applied. Recovery of LDH is almost quantitative (> 98%).

This promised high degree of specificity was confirmed in practice by the one-step isolation of apparently pure LDH from crude extracts of human placenta with a yield of 98–100%. A typical elution profile for such an isolation is presented in fig. 4.

Equivalent purification procedures are clearly possible for other NAD(P)-dependent dehydrogenases, and such procedures are currently being developed for a number of these enzymes. The above "strategy" is, of course only applicable to enzymes with compulsory–ordered mechanisms. For those with random sequential [10] or ping-pong mechanisms, separate affinity chromatography on insolubilized analogues of each of the substrates in turn would presumably be the most effective procedure.

3.1. *Affinity chromatography as a tool for mechanistic studies*

The results described above lend unambiguous support to the compulsory–ordered kinetic mechanism proposed for LDH. The evidence in favour of this mechanism is, of course, already very strong, but the same cannot be said of most other pyridine nucleotide-linked dehydrogenases. Clearly an approach based on affinity chromatography similar to that described here could be of considerable value in this field and, with proper experimental design, should be capable of unequivocally distinguishing between at least the main types of kinetic mechanisms and possibly providing further information about them.

Some such further information regarding ligand-binding by LDH may be derived from the results presented in fig. 3.

Here it is shown (fig. 3 D) that LDH 1 also binds to the insolubilized oxamate in the presence of NAD^+, but relatively weakly, being merely retarded (even at saturating NAD^+ concentrations) in contrast to the very strong retention in the presence of NADH. When 4 mM pyruvate is included in the irrigant with the NAD^+, the retardation is almost abolished (fig. 3 E). Similar chromatography at various pyruvate and NAD^+ concentrations confirms that the counter-effect of pyruvate is due to direct competition with the insolubilized oxamate. These results are in accord with the conclusions drawn from previous kinetic and equilibrium-binding studies [12–15] attributing the substrate-inhibition of LDH by high pyruvate concentrations to the formation of an "abortive" ternary complex of pyruvate with LDH-NAD^+, as illustrated in fig. 1.

The comparative weakness of the chromatographic binding in the presence of NAD^+, as compared with NADH, is consistent with kinetic studies with pyruvate and with free oxamate [9]. None of these studies indicate whether the difference in the binding strengths is attributable to a difference in the induction of the oxamate/pyruvate binding site, or to some other (e.g. steric hindrance) effect related to conformational differences between the oxidized and reduced forms

of the nicotinamide ring. However, the results discussed below, which indicate a positive influence of the nicotinamide moiety on the oxamate/pyruvate binding site, would seem to favour the first possibility.

The recent kinetic and X-ray diffraction studies of McPherson [4] have shown that the binding of NAD(H) to LDH is mainly attributable to the "AMP half" of the molecule since the "nicotinamide half" (represented by NMN^+ and NMNH) does not bind at all to the enzyme in the absence of AMP, and only very weakly in its presence. This is taken as indicating that the AMP end binds first and induces a binding site for the "nicotinamide end". It has not been established, however, whether the AMP half also induces the pyruvate/oxamate binding-site, or whether the nicotinamide end is necessary for this induction (cf. [16]).

The results presented in fig. 3 F and G indicate that there is no binding of LDH to the insolubilized oxamate in the presence of near-saturating concentrations of either 5'-AMP or ADP, indicating that the induction of the pyruvate/oxamate site is indeed absolutely dependent on the mediation of the nicotinamide end of the pyridine nucleotide. It was not possible to cross-check this conclusion by the obvious experiment, chromatography of LDH in the presence of AMP + NMNH, owing to the prohibitive cost of NMNH at the high concentrations which would have been necessary for saturation, but it seems permissible to conclude that LDH ligand-binding follows the totally compulsory order: AMP half, followed by nicotinamide end, followed by pyruvate or oxamate, each ligand in turn inducing the binding site for the next.

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