AFFINITY CHROMATOGRAPHIC DIFFERENTIATION OF LACTATE DEHYDROGENASE ISOENZYMES ON THE BASIS OF DIFFERENTIAL ABORTIVE COMPLEX FORMATION

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1. Introduction

Lactate dehydrogenase (LDH) has a compulsory-ordered kinetic mechanism [1,2] which requires that NADH must bind to the enzyme before pyruvate can bind (fig. 1, left hand side). The H-type isoenzyme, but not the M-type, is characterized by substrate inhibition at high pyruvate concentrations [3]. This substrate inhibition has been explained [3] in terms of the formation of a dead-end complex in which NAD^+ acts as an analogue of NADH in promoting the binding of pyruvate (fig. 1, branch on right hand side). The resulting enzyme–NAD^+–pyruvate complex is commonly termed an abortive complex, because no catalysed electron transfer can occur between the two oxidized substrates (in contrast to the 'productive' enzyme–NADH–pyruvate complex).

The negligible substrate inhibition exhibited by the M-type isoenzyme suggests that it forms the abortive complex only to a negligible extent compared to the H-type. It has been suggested that abortive complex formation is a significant metabolic control mechanism, and the different behaviour of the H and M forms has been rationalized in terms of different functional roles for the two isoenzymes [3–5].

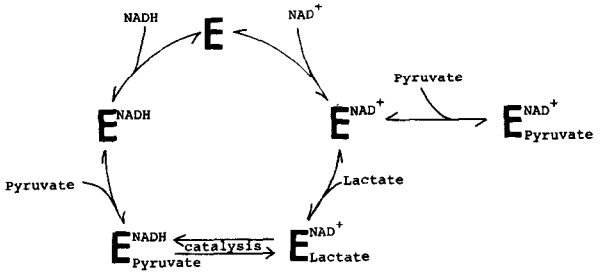

Fig. 1. Kinetic mechanism of lactate dehydrogenase, showing abortive complex formation as a branch on the right-hand side of the diagram (E = lactate dehydrogenase).

In a previous paper [6] we described an effective affinity chromatographic system for LDH based on the affinity of the enzyme for immobilized oxamate, a pyruvate analogue. Owing to the ordered kinetic mechanism, adsorption of the enzyme by the immobilized oxamate gel does not occur unless NADH is included in the applied enzyme samples and in the irrigating buffer.

We show here that when NAD^+ rather than NADH is included in the irrigating buffer, the H and M forms display differential binding to the immobilized oxamate consistent with the proposed differences in their ability to form the abortive complex. The M form shows only very marginal affinity in the presence of NAD^+, while the H form is significantly retarded, though much more weakly than in the presence of NADH. This weak 'abortive complex affinity' can be usefully re-inforced by careful choice of conditions, and the different behaviour of the two isoenzymic forms may then be utilized to allow the specific isolation of the M_4 isoenzyme in the course of the affinity chromatographic purification of LDH from crude tissue extracts.

2. Experimental

The M_4 isoenzyme (LDH-5) and the H_4 isoen-

zyme (LDH-1), both from hog tissues, were generous gifts donated by Boehringer, Mannheim. Other materials and the chromatographic procedures were as previously described [6].

The method of preparing the immobilized oxamate gel is based on carbodiimide promoted coupling of oxalate to the terminal amino group of aminohexyl Sepharose [6]. We have since found that the method as originally described [6] gives somewhat erratic results and relatively low substitution levels owing, apparently, to decomposition of a proportion of the carbodiimide-activated oxalate. This has also been recently observed by Spielmann et al. [7]. In the original coupling procedure, the starting pH was 4.7 and was not controlled during the course of the reaction. The pH rises as the coupling proceeds and although the original method generally yields effective gel preparations, more consistent results are obtained if the coupling is carried out at a controlled pH of about 7.5. Further increase in substitution levels can be achieved by increasing the concentrations of potassium oxalate to 244 mg (dissolved in 1 ml water) per ml of gel, and of water-soluble carbodiimide to 148 mg (dissolved in 0.3 ml water) per ml of gel.

3. Results and discussion

As demonstrated previously [6], both the H and M forms of LDH are very strongly and specifically retarded by columns of immobilized oxamate in the presence of NADH, and are eluted only when the NADH is omitted from the irrigant (fig. 2B). Non-biospecific interaction of the LDH with the spacer-arm assembly complicates this picture unless a high electrolyte concentration is maintained in the irrigant. With gels prepared as previously described [6], 0.3 M KCl or NaCl is just sufficient to overcome such interference but 0.5M salt was routinely used to ensure elimination of non-biospecific absorption effects.

Under these conditions, when NADH is replaced by NAD^+ in the irrigating buffer the H form is weakly retarded but there is no detectable retardation of the M form (fig. 2C). This is consistent with the view (based on kinetic studies) that only the H isoenzyme forms the abortive LDH–NAD^+–pyruvate complex to a significant extent and the weakness of the retardation of this isoenzyme is consistent with the relative weakness of the abortive interaction.

The retardation of the H form in the presence of NAD^+ is enhanced if the chromatography is carried out in the cold (2–5°C) rather than at room temperature (15–20°C). Also, as shown in fig. 2D, the abortive binding is very considerably reinforced if the salt concentration in the irrigant is decreased. Optimum results are obtained if the salt concentration is adjusted to a threshold level below which non-biospecific adsorption just begins to become troublesome. With the low-substitution gels used in the experiments illustrated in fig. 2, the threshold KCl concentration was about 0.3 M. At this salt concentration non-biospecific interactions are marginal and not troublesome; both isoenzymic forms remain unretarded in the absence of pyridine dinucleotide but the H_4 form is now relatively strongly adsorbed in the presence of NAD^+ and is cleanly eluted when the NAD^+ is discontinued (fig. 2D).

This apparent strengthening of the weak 'abortive complex affinity' at lower salt concentrations is similar to effects described elsewhere for other weak bioaffinity systems [8]. It has been suggested that this strengthening is due to a 'compound affinity' effect in which the threshold salt concentration allows the operation of marginal non-biospecific interactions with the spacer arm which synergistically reinforce the weak bioaffinity to produce a relatively strong retardation, without being sufficient to cause any significant retardation on their own [8,9].

This interpretation appears to be borne out by other evidence showing that replacement of the hydrophobic spacer-arms with hydrophilic ones abolishes the re-inforcement effect, leaving only the slight retardation attributable to the weak bioaffinity even when KCl is omitted altogether from the irrigating buffer. (P. O'Carra, S. Barry and T. Griffin, unpublished).

With the gels under consideration here (i.e. those containing the hydrophobic hexyl spacer-arm), the degree of non-biospecific interaction increases as the level of substitution of the gel with spacer-arm and ligand is increased. With the more highly substituted affinity gels, prepared from highly-substituted aminohexyl–Sepharose by the modified method of oxalate coupling described in the Experimental section, higher threshold salt concentrations are required – typically 0.5–0.7 M KCl, the exact concentration required

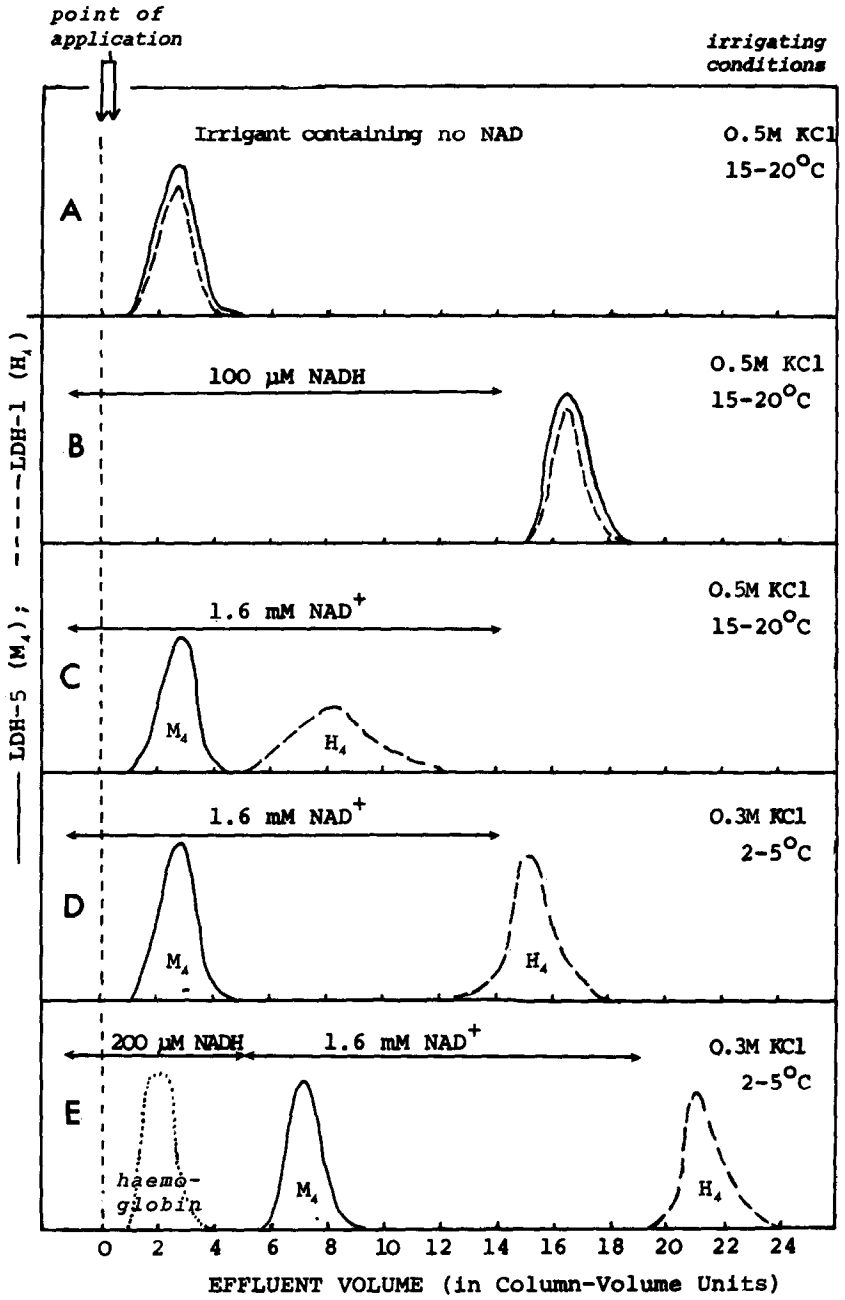

Fig. 2. Model affinity chromatographic studies of the M_4 and H_4 isoenzymes, using a lightly-substituted immobilized oxamate gel. The irrigating buffer consisted of 0.05 M potassium phosphate buffer, pH 6.8, containing the KCl and pyridine nucleotide additions indicated. The column volume (i.e. the operating volume of the bed of affinity gel) was 2 ml; 0.4 ml samples were applied to the column and 0.4 ml fractions were collected using a Gilson microfractionator. Other details of the chromotographic procedure were as previously described [6]. 0.05–0.2 ml aliquots of the fractions were assayed for LDH activity by a modification [6] of the method of Fritz et al. [13]. Separate elution profiles for the two isoenzymes were obtained by chromatographing the isoenzymes separately and then together under identical conditions. In the chromatographic run illustrated in E, haemoglobin was included in the applied sample as a representative 'non-specific' protein to mark the elution position of such proteins. It was monitored in the effluent by spectrophotometric assay of the absorbancy at 417 nm due to the Soret band.

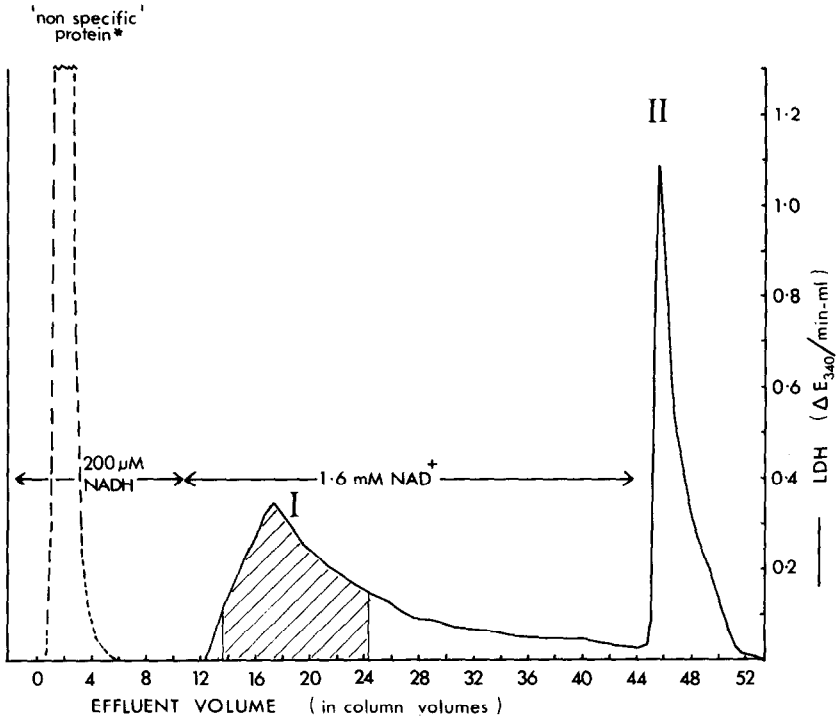

Fig. 3. Isolation of the M_4 isoenzyme from a crude human placental extract. Frozen human placenta was washed with saline and homogenized in an equal volume of 0.1 M potassium phosphate, pH 7.0 (Sorval omni-mixer setting 8, 1 min). The homogenate was centrifuged at 35 000 *g* for 30 min. The supernatant was made up to 0.5 M KCl and 400 μM NADH and adjusted to pH 6.8 with 0.5 M KOH. A 0.4 ml aliquot was applied to a column of immobilized oxamate gel (1.2 ml bed volume), which had been previously irrigated with one column-volume of 0.05 M potassium phosphate buffer, pH 6.8, containing 200 μM NADH and 0.5M KCl. After application of the sample, the column was irrigated first with buffer containing 200 μM NADH and 0.5 M KCl (until the band of non-specific protein was washed through). Then the NADH was replaced by 1.6 mM NAD^+ as indicated. Both the extraction procedure and the chromatography were performed at 2–5°C. The breakthrough peak of 'non-specific protein' was monitored originally by the Lowry method [10] but subsequently by following individual proteins, such as haemoglobin and myoglobin monitored spectrophotometrically at 417 nm, as in this experiment. The elution of these latter proteins coincides almost exactly with the peak monitored by the Lowry method.

varying somewhat for each separately prepared batch of affinity gel.

With these highly substituted gels even the M_4 form of LDH shows a weak NAD^+-dependent retardation at the threshold salt concentration and this retardation becomes quite strong if the salt concentration is decreased slightly below the threshold level. This suggests that the M-type isoenzyme also forms the abortive complex, though much more weakly than the H-type. This is not inconsistent with the kinetic evidence [3,4].

The reinforced 'abortive complex affinity' may be used to specifically resolve the M_4 isoenzymic form in the course of the affinity chromatographic purification of LDH. The tactic adopted is demonstrated in the model chromatographic run illustrated in fig. 2E. and in the separation illustrated in fig. 3. An NADH-containing buffer is used for the application of the enzyme and for the initial irrigation so that both H and M forms bind strongly and are separated from 'non-specific protein' which elutes straight through. The NADH is then replaced by NAD^+ in the irrigant and the M_4 isoenzyme is eluted. The H_4 form remains bound and is subsequently eluted when the NAD^+ is discontinued in the irrigant.

Tissue extracts from which LDH is isolated generally contain, in addition to the H_4 and M_4 forms, the hybrid forms H_3M, H_2M_2 and HM_3. These hy-

brids all contain H-type subunit(s) and, as expected, they display retardation due to 'abortive complex binding' but this retardation is weaker than that shown by the H_4 isoenzyme and the hybrid forms tend to leak slowly off the column, particularly the M_3H form. As mentioned, above, the M_4 form itself displays a very weak abortive complex affinity and this can further interfere with the separation if the salt concentration is decreased below the threshold level. For these reasons the threshold salt concentration must be carefully adjusted if contamination of the M_4 form by the hybrids is to be avoided. The bulk of the hybrid material is eluted together with the H_4 form when the NAD^+ is discontinued.

Fig. 3. illustrates the specific isolation and purification of the M_4 isoenzyme from a crude extract of human placenta. The gel used in this particular experiment was prepared by the modified coupling procedure described in the experimental section and was of a higher substitution level than the gel preparations used for the model studies illustrated in fig. 2. It therefore required a higher 'threshold' salt concentration, 0.5 M KCl being found to give the best results. As noted previously [6] the bulk of the protein in the crude extract is washed straight through the column after application of the sample in NADH, and any non-LDH protein which becomes adsorbed remains adsorbed after subsequent elution of the LDH. Peak I, eluted after the change-over to NAD^+ (fig. 3), was found to contain only the M_4 isoenzyme as judged by the urea-inhibition procedure of Emery [11] and by polyacrylamide gel electrophoresis [12]. Electrophoresis of peak II showed that it contained the H_4 isoenzyme and the three hybrids isoenzymes. Recovery of the applied LDH was about 98%, slightly over half of this being the M_4 isoenzyme of peak I. This seems to represent near-quantitative recovery of this isoenzyme (C. M. Spellman and P. F. Fottrell, personal communication) and the isolated product remains remarkably stable, losing very little activity when stored at 2–5°C for several weeks in the NAD^+-containing buffer in which it is eluted.

This contrasts with the low recovery and the very poor stability of the M_4 isoenzyme from human placenta when isolated by conventional ion-exchange chromatography (C. Spellman and P. F. Fotrell, personal communications). This stabilization, however may be attributed to the efficiency and rapidity of the affinity chromatographic procedure in general rather than to the modification allowing specific separation of the M_4 isoenzyme. The M_4 enzyme can also be isolated as a stable preparation and in good yield by first isolating the total LDH by the affinity chromatographic method as previously described [6] and subsequently seperating the isoenzymes by ion-exchange chromatography [13].

The affinity chromatography illustrated in fig. 3 represents a very small-scale analytical run, but scaling-up of the method for preparative isolation of LDH presented no particular problems. For example 1500 units of LDH from 80 ml of crude placental extract were isolated in pure form in one run on a 10 ml column of a relatively highly substituted gel (prepared by the modified procedure described in the experimental section). During application of the 80 ml sample (containing NADH and KCl) no leakage of LDH from the column was detected and indications are that much larger volumes of extract may be applied to such columns. The application of the sample and the elution of the M_4 isoenzyme should be carried out at 2–5°C, as indicated above. However, elution of the other isoenzymes (peak II, fig. 3) after final discontinuation of the pyridine nucleotide can be carried out at room temperature. Tailing of this LDH fraction, which is sometimes a nuisance when the elution is performed at the lower temperature, is not observed if the column is allowed to warm to room temperature (15–20°C) before discontinuation of the pyridine nucleotide. This tactic also ensures cleaner elution where it is decided to omit the NAD^+-dependent abortive complex step in the elution procedure and to simply elute the total LDH by discontinuation of the NADH. At this stage in the chromatography the LDH is quite stable over the short period required for elution at room temperature, although it is quite unstable in the crude placental extract.

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