Dye–ligand affinity partitioning of lactate dehydrogenase isoenzymes

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ABSTRACT

Aqueous two-phase systems consisting of dextran and polyethylene glycol (PEG) were used to study the partition behaviour of isoenzymes of lactate dehydrogenase (LDH; E.C. 1.1.1.27) from rabbit tissues in the presence and absence of a series of triazine dyes covalently coupled to PEG. The variations in the primary structures of LDH1(H_4) and LDH5(M_4) are reflected by significantly different partition coefficients. A class of dyes exhibiting defined structural elements is able to distinguish between both of these isoenzymes. This may be based on differences in the binding affinity to the catalytic site of the enzyme. The difference in the relative affinities of LDH1 and LDH5 to Procion Blue H-5R, as estimated by affinity partitioning, were corroborated by chromatographic experiments. Affinity partitioning in aqueous two-phase systems can be used to predict and to optimize conditions for the fast and simple chromatographic separation of isoenzymes.

INTRODUCTION

The importance of the determination of isoenzymes is a result of their emergence throughout evolution and their functional significance in existing species. Isoenzymes are linked with cell differentiation and development, and with metabolic regulation and tumour growth. The detection of tissue-specific forms of human isoenzymes improves the specificity and sensitivity of enzyme diagnostic methods [1].

Many approaches are available for the determination of isoenzymes, either by using separation techniques based on differences in the charge of the enzymes and in the affinity to specific immobilized ligands, or by applying non-separating methods based on alterations in the kinetics and physico-chemical and immunological properties of the isoenzymes.

For the separation of lactate dehydrogenase (LDH) isoenzymes, electrophoretic methods [2], anion-exchange chromatography [3] and affinity chromatography using diverse affinity ligands (adenosine-5′-mono-phosphate, oxomate or Cibacron Blue F3G-A) have been described [4–6].

Non-separating detection methods for LDH isoenzymes are based on differences in the stability, kinetic behaviour or immunological properties of the H and M type of the LDH subunits [7].

It is well established that dye–ligand affinity partitioning in aqueous two-phase systems is an attractive approach for studying dye–protein interactions in more detail. The high sensitivity in the recognition of even weak ligand-protein interactions and simple handling particulary favour this method [8–10].

For characterizing the behaviour of enzymes in affinity partitioning, the difference in the logarithms of the partition coefficients in systems with and without the ligand ($\Delta\log K$) is a valuable parameter. The dependence of $\Delta\log K$ on the ligand concentration in a two-phase system generally follows a saturation curve from which two parameters, namely, the maximum partition effect ($\Delta\log K_{max}$) and the relative affinity of the ligand to the protein ($0.5\,\Delta\log K_{max}$) can be calculated [11]. The change in the partition coefficient of an enzyme in the presence of a ligand polymer and competing effectors gives information on the specificity and mode of interaction of the ligand–protein binding [8].

In this work affinity partitoning in aqueous two-phase systems consisting of polyethylene glycol (PEG) and dextran was used to study the binding behaviour of LDH isoenzymes to different triazine dyes covalently coupled to PEG. The aim of this work was to prove the potential of dye–ligands to recognize differences in the properties between isoenzymes determined by individual gene loci.

EXPERIMENTAL

Materials

Lactate dehydrogenase (E.C. 1.1.1.27) from rabbit muscle (LDH5, M_4-isoenzyme) and rabbit heart (LDH1, H_4-isoenzyme), nicotinamide–adenine dinucleotide, oxidized (NAD^+), nicotinamide–adenine dinucleotide, reduced (NADH) and pyruvate were obtained from Sigma (France). PEG 6000, dextran 500 and 2-oxobutyric acid were purchased from Serva (Germany). The dyes were obtained from ICI Organics Division (Blackley, UK) and Ciba-Geigy (Basle, Switzerland). The Vilmax Dye I and Vilmax Dye II were a generous gift from Dr. Mazza, Vilmax (Buenos Aires, Argentina). All other biochemicals were of analytical-reagent grade.

Methods

Preparation of the dye derivatives. The triazine dyes were coupled to PEG 6000 under aqueous alkaline conditions and the reaction product was purified by extraction with chloroform and ion-exchange chromatography on DEAE cellulose (Serva) according to the method of Johansson [9]. The purity of the conjugates was determined by thin-layer chromatography on silica plates G 60 (Merck, Germany) in butan-1-ol–propan-2-ol–ethylacetate–water (20:35:10:35). Procion Blue H-5R and Procion Red HE-3B were coupled to Sepharose 4B (Pharmacia, Sweden), as described by Hughes *et al.* [12].

Enzyme assay. The activities of LDH1 and LDH5 were assayed at 25°C as described by Bergmeyer [13] in 50 mM sodium phosphate buffer solution, pH 7.5, using 0.6 mM pyruvate and 0.18 mM NADH as substrates. The 2-hydroxybutyrate dehydrogenase (HBDH) activity of the LDH isoenzymes was determined in 50 mM

sodium phosphate buffer, pH 7.5, with 3.3 mM 2-oxobutyrate and 0.2 mM NADH at 25°C. One unit of the activity is defined as the amount of the enzyme catalysing the conversion of 1 μmol/min of the respective substrate at 25°C.

Aqueous two-phase partitioning. The two-phase systems were prepared from stock solutions of PEG 6000 (20% w/w), dextran 500 (30% w/w), sodium phosphate buffer, pH 0.5 (0.5 M) and NAD$^+$ (15 mM) by weighing the respective amounts to obtain systems of total mass 2 g with compositions as indicated in the figure legends.

The content of the dye–ligand PEG expressed as percentage is related to the total mass of PEG present in the system. A sample of 2 g of a two-phase system containing about 5 units of LDH was maintained at 25°C and equilibrated by gentle mixing for 30 s. After centrifugation at 1500 × g for 5 min, the samples were withdrawn from both phases and diluted with the test buffer. An inhibition of the enzyme in the assay mixture by the dye–PEG was avoided by dilution of the samples.

The partition coefficient, K, is defined as the ratio of the enzyme activity per unit volume in the top and bottom phases.

Affinity chromatography. Disposable columns (8 mm × 30 mm I.D.) from Bio-Rad Labs. (France) containing a 5-ml bed volume of dye–ligand Sepharose 4B were equilibrated with 50 mM sodium phosphate buffer, pH 7.5, at 25°C. The dialysed enzymes were applied successively to the column. The unbound protein was removed by washing with equilibration buffer at a flow-rate of 10 ml/h. The bound isoenzymes were eluted from the column by a linear gradient of NAD$^+$ (0–3 mM). Fractions of 1 ml were collected and assayed for LDH and HBDH activity.

The concentration of NAD$^+$ in the fractions was determined by measuring the absorbance at 260 nm using a molar absorption coefficient of 17.6 l mol^{-1} cm^{-1}.

RESULTS

Affinity partitioning

Affinity partitioning of LDH1 and LDH5 was screened with a series of dyes and the results are listed in Table I. In the absence of the dye–ligand PEG both isoenzymes partition in favour of the dextan-rich bottom phase ($K < 0.5$), but with different extents. After the addition of 2% dye–PEG to the systems, the partition of the isoenzymes changes depending on the nature of the dye–ligand and isoenzyme, as shown in Table I.

The greatest difference in the affinity partitioning effect (Δlog K) for LDH5 and LDH1 calculated from the data in Table I was found with Procion Blue H-5R, followed by Cibacron Red 3BA, Cibacron Blue F3G-A and Vilmax Dye I. The structures of these dyes are shown in Fig. 1.

To study these dye–LDH interactions in more detail, the partition of LDH1 and LDH5 was studied as a function of the concentration of the dye–PEG. Hyperbolic curves were obtained for both isoenzymes, as exemplified in Fig. 2 with Procion Blue H-5R and Procion Red HE-3B.

The maximum effect of the affinity partitioning (Δlog K_{max}) and the relative affinity (*aff*$_R$ at 0.5 Δlog K_{max}) determined from double reciprocal plots of Δlog K *versus* the dye concentration are listed for selected dyes in Table II. In general, higher Δlog K_{max} values were obtained with LDH5 compared to LDH1. For LDH5 the highest Δlog K_{max} was determined for Procion Blue H-5R. Cibacron Blue F-3GA

TABLE I

AFFINITY PARTITIONING OF LDH ISOENZYMES

Systems (2 g) containing 9% (w/w) dextran 500, 6% (w/w) PEG 6000 (2% dye–PEG), 50 mM sodium phosphate buffer, pH 7.5, and 5 units of LDH1 and LDH5, respectively, were equilibrated at 25°C.

Dye–PEG	Log K	
	LDH1	LDH5
Procion Red HE-3B	+0.894	+1.195
Procion Red MX-8B	−0.353	−0.213
Procion Red H-3B	−0.109	+0.095
Procion Red H-8BN	−0.299	+0.180
Procion Red MX-5B	−0.113	+0.216
Cibacron Red 3BA	+0.146	+1.185
Procion Scarlet MX-G	−0.061	−0.010
Procion Blue MX-G	+0.985	+1.528
Procion Blue MX-3G	+0.320	+0.532
Procion Blue H-5R	+0.786	+2.008
Procion Blue MX-2G	+0.199	+0.548
Cibacron Blue F3G-A	+0.605	+1.430
Procion Navy HE-R	+1.196	+1.557
Procion Navy MX-RB	+0.146	+0.471
Procion Yellow HE-3G	+1.379	+1.445
Procion Yellow H-ER	+0.843	+1.133
Procion Yellow H-A	−0.343	−0.775
Procion Orange MX-G	−0.401	−0.651
Procion Orange MX-2R	−0.135	+0.323
Procion Orange H-2R	−0.124	+0.161
Procion Brown H4-GR	+0.162	+0.126
Procion Green H-4G	+1.603	+1.780
Vilmax Dye I	+0.455	+1.327
PEG alone	−0.411	−0.990

generated the highest Δlog K_{max} with LDH1. With the exception of Procion Red HE-3B, all the other dyes show a significantly higher relative affinity to LDH5 compared to LDH1. The greatest difference in the relative affinity (ten-fold) was found for Procion Blue H-5R.

To analyse the relationship between the enzyme–dye interaction and the binding specificity, the competitive effect of NAD^+ on the affinity partitioning of LDH isoenzymes was studied.

The coenzyme partitions in the aqueous two-phase system used with a K-value of 0.85, independent of the presence or absence of the dye–ligand. As a result of the different affinities of the dye–ligand to both isoenzymes the competition of NAD^+ must be related to a definite concentration of dye–PEG which was defined as the two-fold concentration of the dye–PEG generating 0.5 Δlog K_{max}.

With increasing concentrations of NAD^+ the Δlog K value for both isoenzymes decreased, as shown for Procion Blue H-5R and Procion Red HE-3B in Fig. 3. In contrast to the result with Procion Red HE-3B-PEG, significant differences in the extent of the decrease of Δlog K for LDH1 and LDH5 were observed using Procion Blue H-5R-PEG.
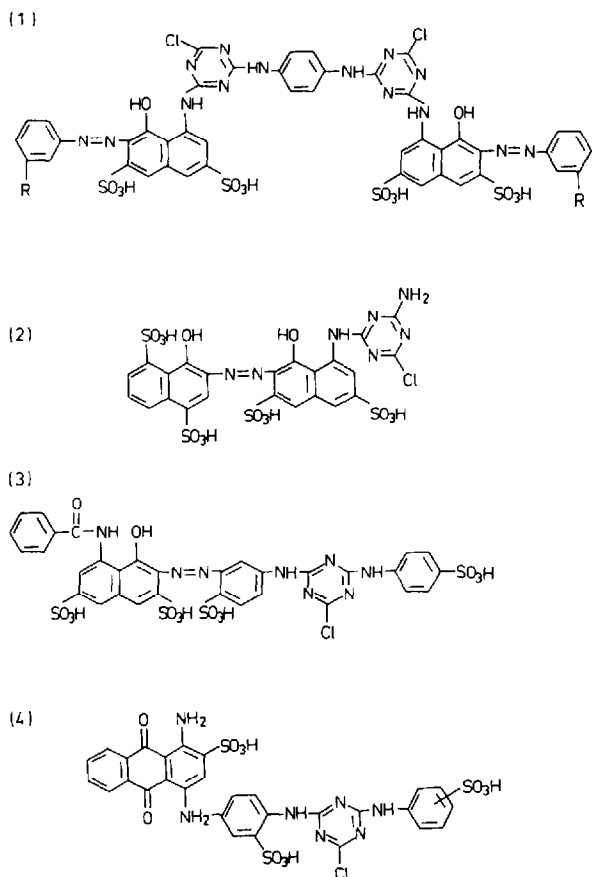
Fig. 1. Structures of triazine dyes: 1 = Procion Red HE-3B (R = SO₃H); Vilmax Dye I (R = H); 2 = Procion Blue H-5R; 3 = Cibacron Red 3BA; 4 = Cibacron Blue F3G-A.

In Table III the data for the effect of NAD^+ on the affinity partitioning of the LDH isoenzymes in the presence of several dyes are listed. In the case of LDH1, NAD^+ competes most effectively with Vilmax Dye I and Cibacron Red 3BA. With LDH5 the competitive effect of NAD^+ was less pronounced for all dyes tested and showed, with the exception of Procion Blue H-5R at 2 mM NAD^+, negligible differences between all dye–ligands.

Affinity chromatography

Applying the results of affinity partitioning in affinity chromatography, a separation of LDH isoenzymes (LDH1 and LDH5) should be realized under certain conditions. In Fig. 4 the elution profile of a mixture of LDH1 and LDH5 on Procion Blue H-5R–Sepharose with increasing concentration of NAD^+ is shown. Two distinct fractions with LDH and HBDH activity appeared. By comparing the ratio of the HBDH:LDH activity in fractions 1 and 2 (0.51 and 0.09, respectively) with those of the individual enzymes it becomes evident that fraction 1 contains LDH1 and fraction 2
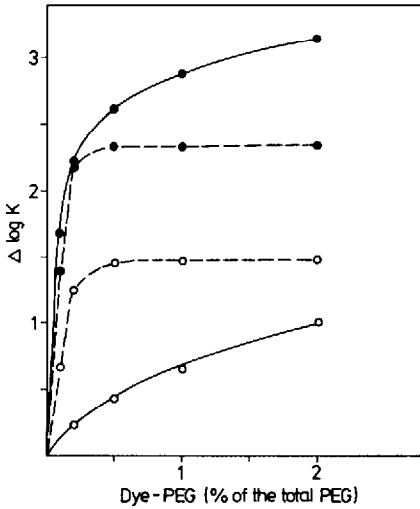
Fig. 2. Affinity partitioning of LDH1 (○) and LDH5 (●) as a function of the concentration of Procion Red HE-3B–PEG (– – –) and Procion Blue H-5R–PEG (———). The systems (2 g) contained 9% (w/w) dextran 500, 6% (w/w) PEG 6000 (partially replaced by dye–PEG), 50 mM sodium phosphate buffer, pH 7.5, and 5 units of the respective isoenzyme. The systems were equilibrated at 25°C.

LDH5 in the pure state. The concentration of NAD^+ in the main fraction of peak 1 and peak 2 was 0.35 and 1.45 mM, respectively. The overall recovery of the LDH and the HBDH activity was 90%.

In accordance with the results of affinity partitioning the affinity chromatography of LDH1 and LDH5 on immobilized Procion Red HE-3B and NAD^+ as eluting effector revealed only one peak containing 91% of the overall LDH and HBDH activity applied. The concentration of NAD^+ in the main fraction was 0.67 mM (Fig. 5).

TABLE II

AFFINITY PARTITIONING OF LDH ISOENZYMES AS A FUNCTION OF THE AMOUNT OF DYE–PEG IN THE SYSTEM

Systems (2 g) containing 9% (w/w) dextran 500, 6% (w/w) PEG 6000, 50 mM sodium phosphate buffer, pH 7.5, and 5 units of enzyme were equilibrated at 25°C.

Dye–PEG	LDH1		LDH5	
	$\Delta\log K_{max}$	$aff_R{}^a$	$\Delta\log K_{max}$	$aff_R{}^a$
Procion Red HE-3B	1.47	0.15	2.34	0.10
Procion Blue H-5R	1.33	1.02	3.33	0.10
Cibacron Blue F3G-A	1.61	0.65	2.45	0.12
Cibacron Red 3BA	1.43	2.38	2.86	0.44
Vilmax Dye 1	1.25	0.92	2.86	0.31

[a] The term relative affinity (aff_R) is defined as that dye–PEG concentration (expressed as % of total PEG) which generates affinity partitioning of 0.5 $\Delta\log K_{max}$.
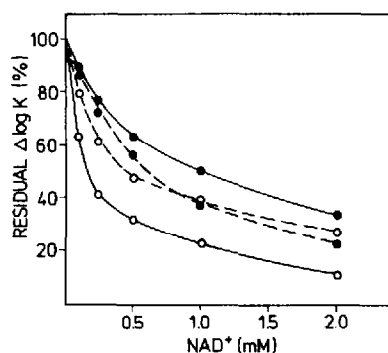
Fig. 3. Affinity partitioning of LDH isoenzymes as a function of the amount of NAD$^+$ added to the system. The systems (2 g) contained 9% (w/w) dextran 500, 6% (w/w) PEG 6000, including 0.3% Procion Red HE-3B–PEG (– – –) and 2% Procion Blue H-5R–PEG (————) for LDH1 (○) and 0.2% Procion Red HE-3B–PEG and 0.2% Procion Blue H-5R–PEG for LDH5 (●), 50 mM sodium phosphate buffer, pH 7.5, and 5 units of the respective isoenzyme. The systems were equilibrated at 25°C.

DISCUSSION

Aqueous two-phase systems consisting of dextran and PEG are able to recognize differences in the primary structures of both LDH isoenzymes. The higher log K value for LDH1 in comparison to LDH5 may be caused by different surface properties (a larger negative net charge or degree of hydrophobicity) of this isoenzyme, which is also indicated by the existence of different epitopes on LDH1 and LDH5 [6].

The interaction of triazine dyes with multiple forms of enzymes, including isoenzymes, as shown for alkaline phosphatase, collagenase, creatine kinase and LDH, is preferentially studied by kinetic and chromatographic methods [14–17].

The applicability of dye–ligand affinity partitioning for this study was restricted.

TABLE III

INFLUENCE OF NAD$^+$ ON THE AFFINITY PARTITIONING OF LDH ISOENZYMES

Systems (2 g) containing 9% (w/w) dextran 500, 6% (w/w) PEG 6000, 50 mM sodium phosphate buffer, pH 7.5, and 5 units of enzyme were equilibrated at 25°C.

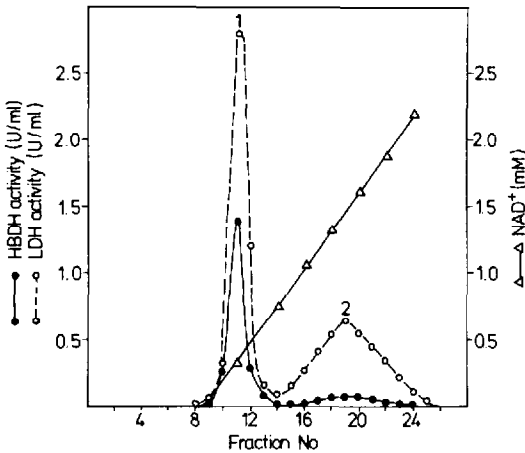| Dye–PEG	Residual Δlog K (%)			
	LDH1		LDH5	
	NAD$^+$ (mM)		NAD$^+$ (mM)	
	0.25	2.00	0.25	2.00
Procion Red HE-3B	62	28	73	22
Procion Blue H-5R	41	11	78	35
Cibacron Blue F3G-A	38	13	64	21
Cibacron Red 3BA	27	0	64	26
Vilmax Dye I	23	0	64	21

Fig. 4. Affinity chromatography of lactate dehydrogenase isoenzymes on Procion Blue H-5R–Sepharose 4B. The column (1.5 cm × 3.0 cm) was equilibrated with 50 mM phosphate buffer, pH 7.5, at 25°C. The dialysed isoenzymes (5 units of LDH activity each) were applied. The enzymes were desorbed by a linear gradient of NAD$^+$ (0–3 mM) in equilibration buffer. The LDH activity (○– – –○) and the HBDH activity (● ●) were determined in all fractions (1 ml).

Ligands such as Cibacron Red 3BA, Procion Blue H-5R, Cibacron Blue F3G-A and Vilmax Dye II covalently coupled to PEG lead to an increase on the difference in the partition coefficients of both isoenzymes, whereas other dyes such as Procion Scarlet MX-G and Procion Yellow HE-3G reduce the difference. Other dyes such as Procion Yellow H-A and Procion Orange MX-G do not show any interaction with either isoenzyme.
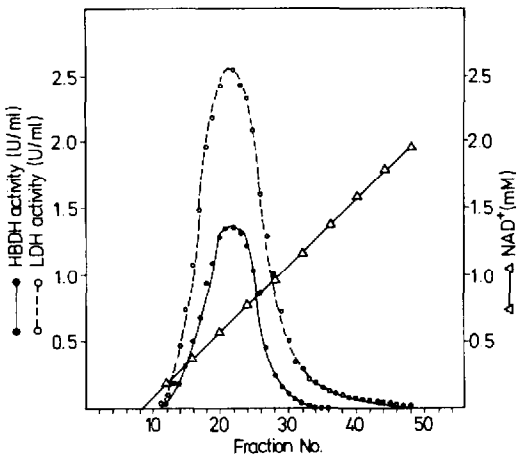

Fig. 5. Affinity chromatography of lactate dehydrogenase isoenzymes on Procion Red HE-3B–Sepharose 4B. The column (1.5 cm × 3.0 cm) was equilibrated with 50 mM phosphate buffer, pH 7.5, at 25°C. The dialysed isoenzymes (13 units of LDH activity each) were applied. The enzymes were desorbed by a linear gradient of NAD$^+$ (0–3 mM) in equilibration buffer. The LDH activity (○– – –○) and the HBDH activity (●—●) was determined in all fractions (1 ml).

By comparing the structure of all the dyes investigated with the results obtained, one common feature can be suggested. Only polysulphonated dyes containing 1-amino-8-naphthol-3,6-disulphonic acid rings or 1-amino-4-(4'-aminophenylamino)-anthraquinone-2,3'-disulphonic acid moieties are able to distinguish between both isoenzymes.

For a deeper insight, only dyes which generate high differences in the $\Delta\log K$ values of both isoenzymes have been selected to compare their relative affinity to LDH1 and LDH5. These data have additionally been compared with results obtained with Procion Red HE-3B, a well characterized pseudo-biospecific ligand of LDH from rabbit muscle [6,18]. Generally, LDH5 possesses a higher relative affinity to the dye–ligands selected than LDH1. Only Procion Red HE-3B showed a similar high relative affinity to both isoenzymes. As shown in the competitive experiments with NAD^+, the partition of the isoenzymes in the presence of Procion Red HE-3B, Procion Blue H-5R, Cibacron Red 3BA, Cibacron Blue F3G-A and Vilmax Dye I is based on pseudo-biospecific interactions. The hydrophobic nicotinamide pocket and arginine residues of the substrate binding site are probably involved in the binding of dyes such as Cibacron Blue F3G-A and Procion Red HE-3B with such a high affinity [18,19].

As no exact data about the structural differences in the active centre of LDH1 and LDH5 from rabbit tissues are available at present, the differences in the relative affinity of the dyes to the LDH isoenzymes cannot be discussed on the basis of the specific interaction of amino acid residues with structural elements of the dyes.

From kinetic analysis it becomes obvious that LDH isoenzymes exhibit different catalytic properties. As already published and corroborated by these authors' experiments (data not shown), both LDH isoenzymes possess a higher affinity to NADH than to NAD^+. The differences in the affinity of LDH5 to NAD^+ and NADH are more pronounced than for LDH1. The K_m values for NAD^+ of LDH1 and LDH5 are very similar [13,20]. Consequently, the differences in the decrease of $\Delta\log K$ with increasing concentrations of NAD^+ is more affected by the different affinity of the isoenzymes to the dye–ligand (Procion Blue H-5R and Procion Red HE-3B, respectively) than by the differences in the affinity to NAD^+.

The affinity chromatographic separation of isoenzymes depends on differences in: (1) the affinity between the ligand and the enzymes; (2) the affinity between the enzymes and the competitive effector; and (3) the classes of binding sites. Considering these features and the results of affinity partitioning, only Procion Blue H-5R in combination with NAD^+ as a competitive effector should be a good biomimetic ligand for the chromatographic separation of LDH isoenzymes. This is demonstrated by the results obtained here.

The separation of LDH isoenzymes by using high-performance liquid chromatography with immobilized Cibacron Blue F3G-A, as reported by Lowe et al. [21], can be explained by the results reported in this paper. The difference in the relative affinities of LDH1 and LDH5 to the dye–ligand seems to be sufficient to separate both isoenzymes with increasing concentration of NADH.

In summary, affinity partitioning in aqueous two-phase systems is a sensitive and simple method of studying ligand–isoenzyme interactions. The parameters obtained allow determination of the conditions for the chromatographic separation of isoenzymes with high efficiency.

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