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New aspects of dye-ligand affinity chromatography of lactate dehydrogenase applying spacer-mediated beaded cellulose

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ABSTRACT

The interaction of lactate dehydrogenase (LDH, EC 1.1.1.28) with triazine dyes spacer-linked to beaded cellulose was studied. The length of the spacer influences neither the binding capacity of the dye–affinity adsorbent nor the elution of the enzyme by NAD–sulphite. However, the length of the extension arm strongly affects the elution of LDH by salt. The results with beaded cellulose carrying butyl, hexyl and decyl residues point to pure hydrophobic interaction with LDH. The introduction of a terminal amino or carboxylic group in the aliphatic chain changes significantly the binding and elution behaviour of the enzyme. It is assumed that in addition to specific interactions realized by the dye chromophore and specific domains on the surface of the enzyme the spacer arm generates a second type of binding force which points to hydrophobic interactions. The latter might be the result of specific orientation governed by the dye-ligand–protein interaction.

INTRODUCTION

The interaction of lactate dehydrogenase (LDH, EC 1.1.1.28) from bovine heart muscle with Cibacron Blue F3G-A and related reactive dyes has been thoroughly investigated[1]. In addition to Cibacron Blue F3G-A, which is known to act as a pseudo-ligand to the NAD-binding domain of numerous enzymes[2,3], a number of other dyes have been found to bind more or less specifically, often forming stochiometric dye–enzyme complexes[4]. Among diverse methods for studying dye–enzyme interactions, dye-ligand affinity chromatography is one of the most commonly applied technique, for both analytical and preparative purposes.

Owing to the wide variety of commercially available reactive dyes and to the facility to form a covalent bond with the support, a number of polymers such as agarose, cross-linked dextran, polyacrylamide and porous silica beads have proved useful as matrices[5,6]. In addition to the chemical requirements to act with reactive dyes, the matrices should show minor non-specific adsorption of proteins, high porosity and sufficient rigidity to achieve adequate flow-rates in packed columns, and showed to be inexpensive. Recently, beaded cellulose has become more attractive as

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a general support in chromatographic techniques[7]. As shown by Mislovičová *et al.*[8] and in our laboratory[9], this material seems to be well suited for affinity chromatography applying dyes as ligands.

Affinity chromatography of enzymes on dye-liganded matrices is generally carried out by (i) adsorption of the enzyme from the crude material, (ii) washing the gel to remove unbound material and (iii) desorption of the target enzyme either non-specifically by increasing the ionic strength or more specifically by adding competitive effectors. In the case of LDH, the substrate NAD in the micromolar range in the presence of millimolar concentrations of sodium sulphite is able to displace the bound enzyme by forming a high stable ternary NAD–sulphite–enzyme complex[10].

The success of affinity chromatography not only depends on the specificity of the ligand–enzyme interaction but is also governed by the mode of coupling of the ligand to the matrix. As shown by Lowe[6], a spacer of certain chain length was used advantageously in order to increase the mode of specific docking if, for example, the ligand-binding site is hidden in a hydrophobic pocket or the surface of the matrix causes in some manner a steric hindrance of the ligand–enzyme interaction.

We found recently[9] that adsorption and desorption of heart muscle LDH depends not only on the intrinsic properties of the dye-ligand but also on the length of the spacer between the dye and the cellulose matrix. For example, the Procion dyes Scarlet MX-G, Orange MX-G, Yellow HE-3G and Green H-4G exhibited strong interactions with LDH, as demonstrated by affinity partitioning[9], but their respective dye–cellulose matrices revealed no adsorption when the dyes were coupled directly to the resin. However, if the immobilization of the dye was realized via a spacer of sufficient chain length, both the specific adsorption of LDH increased markedly and the conditions for the elution of the enzyme were changed. On the other hand, Cibacron Blue F3G-A and Procion dyes such as Red HE-3B, Red HE-7B, Yellow HE-4R and Navy H-ER showed strong binding even if they were coupled directly to the beaded cellulose.

In order to obtain a deeper insight into the function of the spacer in performing dye-ligand affinity chromatography of LDH, Procion Scarlet MX-G was selected as a model ligand to elucidate the chemical requirements of the dye–enzyme interaction and the influence of the spacer length for optimum fitting of the ligand to the binding centre.

EXPERIMENTAL

LDH was partially purified from bovine heart muscle with 12-fold enrichment as described elsewhere[9]. The enzyme had a specific activity of 40–70 units/mg protein.

The dye derivatives of beaded cellulose (Divicell, VEB Arzneimittelwerk Leipzig, G.D.R.) were prepared according to the method of Lowe and Pearson[11]. The synthesis of dye–(spacer)–beaded cellulose (Fig. 1) was described in a previous paper[9] using N-chlorocarbonyloxy-5-norbornene-2,3-dicarboximide (Cl-CO-ONB) activated beaded cellulose[12] and the respective aminoalkyl dye.

The determination of spacer-mediated dye substituents by acid hydrolysis or by the use of Cadoxen[9] was unsuccessful because of the insolubility of the cross-linked dye–affinity adsorbents. The degree of dye substitution may be assumed to be roughly equal for this series because the same batch of activated cellulose was used and the dye substitutions were performed under identical conditions.

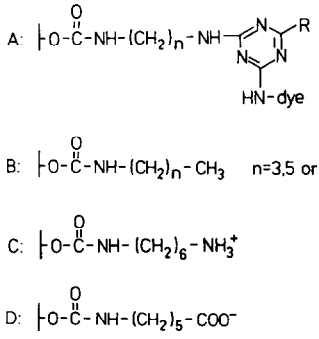


Fig. 1. Structures of dye–spacer–beaded cellulose (A) and of beaded cellulose with different extension arms (B–D).

For the preparation of liganded cellulose as presented in Fig. 1 0.05 mol each of 1-aminobutane, 1-aminohexane (Fig. 1B) and 1,6-diaminohexane (Fig. 1C) were dissolved in 10 ml of distilled water; 1-aminodecane (Fig. 1B) was dissolved in 10 ml of dimethyl sulphoxide–water (1:1, v/v), and ε-aminocaproic acid (Fig. 1D) was dissolved in 20 ml of 0.1 *M* sodium hydrogencarbonate. The solution containing the ligand was adjusted to pH 9 with 5 *M* hydrochloric acid. For coupling, 10 ml of Cl-CO-ONB-activated beaded cellulose were added stepwise to the ligand solution. The suspension was gently shaken at room temperature for 20 h, then the liganded cellulose was filtered off and washed thoroughly with distilled water.

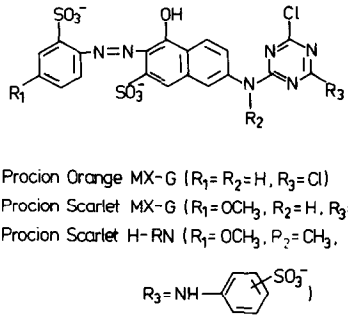
For affinity chromatographic runs, prepurified LDH dialysed against 20 m*M* potassium phosphate buffer (pH 7.0 or 5.5) containing 5 m*M* 2-mercaptoethanol and 1 m*M* EDTA was loaded onto columns (5 × 1 cm I.D.) packed with the respective affinity adsorbent. To remove unbound protein the resin was washed extensively with the same buffer. The adsorbed LDH was then eluted with buffer containing either 1 *M* potassium chloride or 0.05 m*M* NAD plus 1 m*M* sodium sulphite. The main fractions of enzyme activity were pooled and concentrated by ultrafiltration.

The activities of LDH and malate dehydrogenase (MDH, E.C. 1.1.1.37) were measured spectrophotometrically at 340 nm using the following tests: 0.1 *M* potassium phosphate buffer (pH 7.0), 0.8 m*M* pyruvate for LDH and 0.8 m*M* oxaloacetate for MNH and 0.2 m*M* $NADH_2$. Protein was assayed according to Bradford[13].

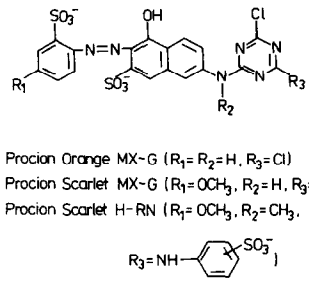
RESULTS

Immobilized Procion Scarlet MX-G and some structurally related dyes (Fig. 2) were studied for their binding to LDH and their elution. None of these dyes was able to interact with the enzyme if they had been coupled directly to beaded cellulose. Immobilizing the ligands via 1,6-diaminohexane attached to the triazine ring (Fig. 1A) resulted in sufficient binding capacities for all dye matrices (Table I). The desorption of LDH was only achieved with NAD–sulphite and not with potassium chloride. No significant differences with respect to yield and purification factor were observed (Table I).

Procion Scarlet MX-G was used as a model ligand in order to study the influence of the spacer on the interaction of LDH. As demonstrated in Table II, no signif-



Procion Orange MX-G ($R_1 = R_2 = H$, $R_3 = Cl$)
Procion Scarlet MX-G ($R_1 = OCH_3$, $R_2 = H$, $R_3 = Cl$)
Procion Scarlet H-RN ($R_1 = OCH_3$, $R_2 = CH_3$,



Procion Orange MX-G ($R_1 = R_2 = H$, $R_3 = Cl$)
Procion Scarlet MX-G ($R_1 = OCH_3$, $R_2 = H$, $R_3 = Cl$)
Procion Scarlet H-RN ($R_1 = OCH_3$, $R_2 = CH_3$,

Fig. 2. Structures of Procion dyes used.

icant differences with respect to the dependence of the binding capacity of the prepurified LDH on the length of the spacer was observed. However, although 80–100% of bound LDH could be eluted specifically by NAD–sulphite independent of the number of methylene groups in the spacer, the length of the extension arm strongly affects the elution of LDH with potassium chloride. The non-specific elution with salt decreased with increasing spacer length of the matrix.

However, the specific activity of the enzyme with 180–200 units/mg and the SDS pattern of the main fractions of LDH eluted with NAD–sulphite were similar with all kinds of spacer length probes. Regarding the binding specificity it is worth noting that about 30% of bound LDH can be eluted from the diaminohexyl- and

TABLE I

BINDING CAPACITY AND AMOUNT OF ELUTED ACTIVITY OF PREPURIFIED LDH FROM DYE–(DIAMINOHEXYL)–BEADED CELLULOSE

The binding capacity of dye–beaded cellulose was determined using prepurified LDH by DEAE-Sephadex chromatography[9]. Columns (5 × 1 cm I.D.) containing 2 g of the respective dye–cellulose were loaded with an excess of LDH at 10°C. The amount of activity adsorbed (defined as 100%) was calculated from the difference in the total activity of LDH loaded onto the column and the unbound LDH determined in the breakthrough fraction and in the wash pool. The purification factor was calculated from the increase in the specific activity of the enzyme. For the elution of LDH, 20 m*M* potassium phosphate buffer (pH 7.0) containing 5 m*M* 2-mercaptoethanol, 1 m*M* EDTA, 0.05 m*M* NAD and 1 m*M* sodium sulphite was used. The specific activity of eluted LDH varied between 130 and 230 units/mg protein, depending on the purity of the starting material.

Procion dye	*Binding capacity (units/g moist gel)*	*Eluted LDH activity (%)*	*Purification factor*
Orange MX-G	400–690	60–70	2.5
Scarlet MX-G	500–770	60–80	3
Scarlet HR-N	400–600	60–80	4
Red MX-8B	500–650	50–70	3
Red H-3B	500–650	70–85	3
Red S6	350–500	70–80	4.5

TABLE II

BINDING AND ELUTION BEHAVIOUR OF LDH ON PROCION SCARLET MX-G-(SPACER)-BEADED CELLULOSE

Experimental details as in Table I. The LDH was desorbed by 1 *M* KCl followed by NAD–sulphite. Before use of NAD–sulphite containing buffer the column was washed free of KCl. Procion Scarlet MX-G-(NH-C_n-NH)COO-cellulose.

n	*Binding capacity (units/g moist adsorbent)*	*Activity of bound LDH (%)*		
		Elution with 1 M KCl	*Followed subsequently with NAD–sulphite*	*Elution with NAD–sulphite alone*
2	310–350	20–60	42–16	80–100
6	350–400	5–20	85–65	80–95
10	350–450	1– 3	95–85	90–100

diaminodecyl-beaded cellulose using ethylene glycol (50%, v/v), but the elution did not occur if affinitity adsorbents with a shorter spacer length were applied.

In Table III the desorption of LDH from Cibacron Blue F3G-A and Procion Red HE-3B coupled either directly to cellulose or via a spacer is shown. The elution of LDH by 1 *M* potassium chloride was significantly hindered if the diaminohexyl spacer between the dye and the cellulose was introduced, whereas the specific elution of LDH with NAD–sulphite was not changed. In addition, no difference in the specific activity were found. The purification of the enzyme was 4–5-fold in both types of dye–affinity matrices.

The different behaviour of directly coupled and spacer-mediated Cibacron Blue F3G-A with respect to the elution of LDH was used to separate LDH and MDH in a crude heart muscle extract. Both affinity adsorbents bound MDH with 2–4-fold higher capacity compared with LDH; 90–95% of the bound activities of MDH and LDH were recovered by desorption with salt from the directly bound dye–absorbent with

TABLE III

DESORPTION OF LDH FROM CIBACRON BLUE F3G-A AND PROCION RED HE-3B DIRECTLY BOUND TO BEADED CELLULOSE (BC) OR ATTACHED VIA A DIAMINOHEXYL SPACER

Experimental details as in Tables I and II.

Adsorbent	*Activity of bound LDH (%)*	
	Elution with 1 M KCl	*Elution with NAD–sulphite*
Cibacron Blue F3G-A–BC	95	95
Cibacron Blue F3G-A-(C_6)-BC	10–30	90
Subsequent elution with by NAD–sulphite	50–80	
Procion Red HE-3B–BC	95	95
Procion Red HE-3B-(C_6)-BC	5–30	70–90
Subsequent elution with NAD–sulphite	30–60	

slow retardation of LDH. Applying spacer-linked Cibacron Blue F3G-A, 85% of bound MDH and only 20% of adsorbed LDH were eluted by 1 *M* potassium chloride, resulting in a 3-fold enrichment of MDH.

To study the function of the spacer for the binding of LDH, three adsorbents with extension arms differing in length and in the terminal group were prepared (Fig. 1). No binding of LDH was observed with adsorbents carrying a hydrophobic butyl residue (Fig. 1B), as studied at pH 7.0 and 5.5. However, LDH was bound by beaded cellulose liganded with hexyl or decyl residues (binding capacity 200–400 units per gram of moist adsorbent). Partial desorption of the enzyme from hexyl- and decylcellulose (45% and 11% of the bound LDH, respectively) was achieved only if ethylene glycol-containing buffer (50%, v/v) was used. Potassium chloride and NAD–sulphite were unable to desorb the enzyme.

If a hexylamino residue was coupled to activated cellulose (Fig. 1C), this modified adsorbent bound LDH at both pH 5.5 and 7.0 without a difference in the binding capacity (300–500 units per gram of moist absorbent). Under both conditions 70–100% of the activity can be desorbed by 1 *M* potassium chloride but not by NAD–sulphite or ethylene glycol.

As an alternative ligand, caproic acid was attached to beaded cellulose (Fig. 1D). This adsorbent did not bind LDH at pH 7.0 but showed an interaction with LDH at pH 5.5. Elution of the enzyme occurred specifically with NAD–sulphite (50–60% of the bound enzyme) or with ethylene glycol (20% of the bound enzyme).

DISCUSSION

Triazine dyes of different classes are well established as biomimetic ligands in affinity separation techniques. Owing to the chemical reactivity, most of the dyes have been coupled to matrices by forming a covalent bond with the support via the triazinyl ring of the dye.

In a previous paper[9] we showed that several triazine dyes having the potential to interact with LDH do not bind the enzyme if they are coupled directly to beaded cellulose. The binding of the enzyme to the dyes, however, was achieved by the introduction of a spacer between the matrix and the affinity ligand. Among the group of analogues of Procion Scarlet MX-G no significant changes in the binding capacity, recovery or purity of the enzyme were observed. On changing the spacer length from two to ten methylene groups, the degree of non-specific desorption of LDH with potassium chloride decreased drastically (Table II). This means that in addition to specific interactions realized by the dye chromophore and specific domains on the surface of the enzyme, a second type of binding forces is generated which points to hydrophobic interactions.

This suggestion was confirmed by the results summarized in Table III, in which the elution behaviour of LDH from Cibacron Blue F3G-A– and Procion Red HE-3B–cellulose are directly compared with respect to the mode of dye coupling. Both types of affinity adsorbents were able to bind LDH. However, the introduction of a diaminohexyl spacer diminished the non-specific desorption of LDH by potassium chloride drastically and the main part of the enzyme was desorbed specifically by NAD–sulphite. This allows the assumption that after weakening the specific binding sites by the competitive inhibitor the enzyme might rearrange the conformation in such a manner that the hydrophobic interaction is also abolished.

In contrast, MDH can be displaced by salt from the dye–spacer–cellulose. The introduction of a spacer arm does not retard the enzyme to the same extent as LDH. The results support the findings of Lowe[6] that the increase in the strength of the binding is related to the apparent molecular weight of the protein. In order to establish the intrinsic effect of the spacer on binding and elution of LDH, the matrix was coupled with aminoalkanes of different chain length ($n = 2$–10). The results demonstrate that aliphatic spacers with more than four carbon atoms are able to bind LDH by pure hydrophobic interaction. However, the introduction of a terminal amino group obviously changed the mode of interaction. The enzyme was bound at pH 5.5 and 7.0 and was eluted by potassium chloride but was not desorbed by NAD–sulphite and ethylene glycol. Because of the positive charge of the terminal amino group ($pK \approx 10$), a poor ion-exchange mechanism is assumed to be responsible for the behaviour of LDH. On the other hand, the introduction of a terminal carboxylic group ($pK_{COOH} \approx 4.8$) in the aliphatic chain revealed no interaction of LDH with the liganded cellulose at pH 7.0, probably owing to repulsion of the negative charge of the LDH used, which is composed mainly of isoenzymes 1 and 2. The binding of LDH at pH 5.5 may be caused by electrostatic interaction between the negatively charged carboxylic group and positively charged groups of the enzyme which become dominant at pH 5.5 in addition to hydrophobic interaction. The ability of NAD–sulphite to elute LDH also from this type of adsorbent suggests that the formation of the ternary complexes becomes dominant and weakens both the electrostatic and hydrophobic interactions either directly or indirectly by a conformation change of the protein.

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