STUDY OF POROUS CELLULOSE BEADS AS A DYE-LIGAND MATRIX. EFFECT OF PROTEIN ADMIXTURES AND CONCENTRATION OF IMMOBILIZED DYE IN THE QUANTITATIVE ANALYSIS OF LACTATE DEHYDROGENASE: CIBACRON BLUE INTERACTION

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The effect of protein admixtures and concentration of immobilized dye on the interaction of lactate dehydrogenase (LDH) with Cibacron Blue-bead cellulose has been studied by means of zonal chromatography with Cibacron Blue-dextran T 10 as the mobile ligand. The influence of both variables on the values of dissociation constants of complexes immobilized dye–LDH (K_{I-L}) and mobile dye–LDH (K_{M-L}) can be summarized as follows: The values of K_{I-L} remained practically constant and were found to be lower than those of K_{M-L}. The differences found in K_{I-L} and K_{M-L} values should help to estimate the contribution of nonspecific interaction of the matrix to the total interaction between dye-ligand and the enzyme.

Cibacron Blue 3G-A (C.I. reactive blue 2) and related dyes are sulphonated polyaromatic compounds which bind with considerable specificity and significant affinity to many enzymes and proteins. Fixed to appropriate insoluble supports, they find wide application as affinity adsorbents in dye-ligand affinity chromatography. As stated by Scopes[1], dye-ligand columns in contact with a specific protein (in a specified buffer) may behave in a variety of ways. In the case of Cibacron Blue (CB)-bead cellulose column binding of muscle lactate dehydrogenase (LDH) (refs[2,3]) takes place mostly in the way pointed out under c (ref.[1]). It means that "... the protein binds by interacting biospecifically as well as nonspecifically and can be eluted by inclusion of a natural ligand". This was demonstrated by time–concentration dependence of batch adsorption of LDH on CB-bead cellulose[2]. Later on, in an extensive study, zonal affinity chromatography was used and the results were confirmed[3]. Each of the two mobile ligands, the natural NADH as well as the synthetic CB-dextran T 10, applied separately, was found capable to compete for one binding site of tetrameric LDH with the immobilized dye. The dissociation constant values of the complex LDH: immobilized dye ($0{\cdot}6 \leqq K_{I-L} \leqq 2{\cdot}6$ μmol l^{-1}) established by both methods, indicated, the presence of biospecific interactions. On the other hand, the

differences in the values of dissociation constants K_{I-L} and K_{M-L} (complex LDH: mobile dye) were ascribed to nonspecific interaction with bead cellulose.

The aim of the present work was, under condition of monovalent interaction, to minimize the effect of nonspecific interactions of LDH with the matrix and the effect of other specific proteins in the LDH preparation on the values of dissociation constants K_{I-L} and K_{M-L}. For this purpose CB-bead cellulose with the total concentration of immobilized dye of 138 – 1 155 μmol CB l^{-1} gel and LDH in the form of crude and a purified preparation was used.

EXPERIMENTAL

Dye-Ligand Conjugates

Porous cellulose beads Ostsorb B (Spolchemie, Ústí n/L) had a particle diameter within the range of 100—400 μm giving 12·5% (w/w) dry matter. Dextran T 10 and high-molecular-weight dextran (approx. average molecular weight 2 . 10^6) were from Pharmacia Biotechnology (Uppsala) and Sigma (St. Louis). Cibacron Blue 3G-A (C.I. reactive blue 2) was kindly provided by Ciba-Geigy (Basel). Bead celluloses as well as dextran were derivatized (under base catalysis) with Cibacron Blue (CB) at 80°C (refs[4,5]). The degree of substitution (DS) was determined spectrophotometrically either in water solution (CB-dextran T 10) or in cadoxene solution (CB-bead cellulose) at 610 or 630 nm (refs[3,4]). The CB-cellulose conjugates prepared are reviewed in Table I. Cadmium tris(ethylenediamine) hydroxide (trivial name cadoxene) was prepared by the general procedure after single saturation with CdO (ref.[6]).

Enzyme Purification

The filtered and lyophilized[3] extract from beef flank muscle, containing 7·2 units of lactate dehydrogenase (L-lactate: NAD oxidoreductase, EC 1.1.1.27) (LDH) per mg of solid material, was used as the crude LDH (LDH No. 1). The purified LDH (No. 2, approx. 400 U/mg protein) was prepared by dye-ligand chromatography of the crude material on the Remazol Blue-bead

TABLE I
Review of CB-cellulose beads

No.	Total concentration of immobilized dye	
	μmol l^{-1}	mol mol^{-1}
I	137·6	0·3
II	488·9	1·0
III	810·1	1·6
IV	1 155·0	2·4

cellulose column, subsequent ultrafiltration on UF cells provided with a UM 10 (Amicon, Oosterhout) membrane, dialysis and two-fold dilution with glycerol[7]. The LDH preparations were stored at 4°C (No. 1) and −20°C (No. 2) and loss of activity was not observed at least two-three month during the storage. Purity of LDH was controlled by means of a Pharmacia Biotechnology (Uppsala) FPLC equipment, using a standard prepacked column of Superose 12 HR 10/30. The equipment was pre-conditioned as follows: eluting buffer 50 mM phosphate pH 7, flow rate 30 ml/h, UV absorbance monitoring at 280 nm, room temperature. The chromatograms of crude and purified LDH are presented in Fig. 1. Fractions of 0·5 ml collected in order to measure the catalytic activity of LDH (ref.[4]) (not shown).

Adsorption Isotherm

The maximum enzyme concentration retained by CB-bead cellulose (the binding capacity) was determined by a saturation batch-wise adsorption[8]. An aliquot of the adsorptive (LDH in phosphate buffer) was added to an accurate weight of the adsorbent (dye-cellulose conjugate) and the mixture was shaken in a Vibrotherm MIM (Budapest) thermostat at 25 ± 0·2°C. The moles of enzyme added were in 10-fold excess over those of dye molecules immobilized on bead cellulose. After equilibration, the supernatant was removed and the dye-cellulose conjugate was thoroughly washed with phosphate buffer. The retained LDH was desorbed with a CB-dextran T 10 solution (50—100 μ mol CB l^{-1}) and its activity in the eluate (desorptive) was determined[4]. The concentration of the retained enzyme and the concentration of the accessible dye were calculated in terms of μ mol per l of conjugated matrix. Complete data are presented in Fig. 2.

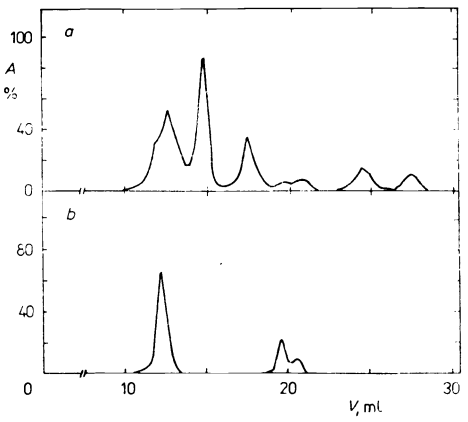

FIG. 1

Elution profiles of crude (*a*) and purified (*b*) LDH were recorded with the aid of the FPLC equipment. Effluent was monitored at 280 nm (ordinate), numbers depicted on the abscissa mean elution volume, *V* ml. The column (Superose 12 HR 10/30) was loaded with 20 U of LDH per 0·3 ml

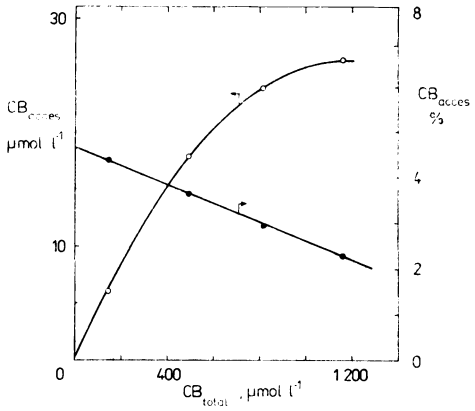

FIG. 2

Saturation adsorption curve of crude LDH plotted as the effect of concentration of total immobilized dye on concentration of accessible immobilized dye expressed in μmol l^{-1} (○) and in % (●)

Zonal Chromatography

Zonal affinity chromatography of LDH was performed at 25°C on immobilized dye column (1·1 cm × 29 cm) equipped with a thermostated jacket[3]. The conjugated cellulose beads used throughout this study are shown in Table I. The conjugated dextran T 10 used as the mobile dye was 104 μmol CB g^{-1}. The results obtained were processed by using the equation valid for monovalent interaction[3,9,10].

RESULTS AND DISCUSSION

The concentration of the accessible immobilized dye of different bead CB-celluloses were determined by the saturation adsorption method[10]. Plot of accessibility of the immobilized dye versus its total concentration showed nonlinear shape (Fig. 2). Crude and purified muscle LDH as the enzyme preparations were applied. The

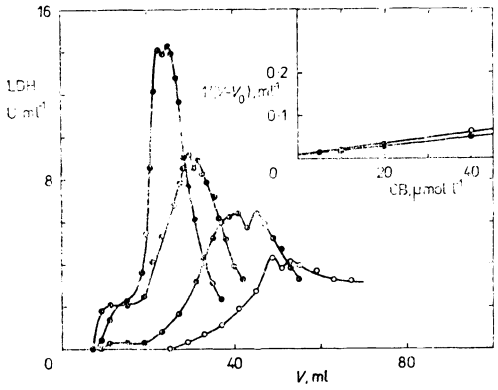

FIG. 3

Zonal elution chromatography of crude LDH on bead CB-cellulose II with varying concentration of CB-dextran T 10 conjugate in the eluting buffer, pH 8·5. 150 U of LDH were applied to the dye column equilibrated with 3 μmol l^{-1} (○), 10 μmol l^{-1} (◑), 20 μmol l^{-1} (◐), and 40 μmol l^{-1} (●) CB-dextran T 10 conjugate each in the eluting buffer. Inset: plot of $1/(V - V_0)$ vs concentration of CB-dextran T 10 according to the equation valid for monovalent interactions[3,9,10], correlation coefficient $r = 0{\cdot}988$. Double peaks in chromatograms were reflected in two set of points and two lines within inset

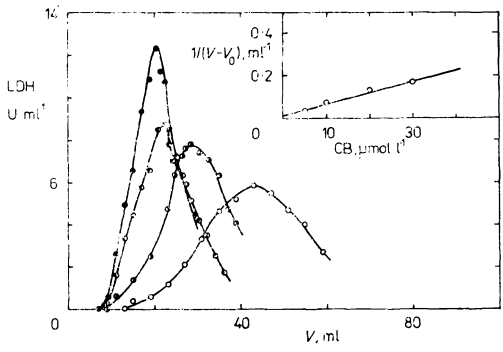

FIG. 4

Zonal elution chromatography of purified LDH on bead CB-cellulose II with varying concentration of CB-dextran T 10 conjugates in the eluting buffer, pH 8·5. 150 U of LDH were applied to dye-columns equilibrated with 5 μmol l^{-1} (○), 10 μmol l^{-1} (◑), 20 μmol l^{-1} (◐), and 30 μmol l^{-1} (●) CB-dextran T 10 conjugates each in the eluting buffer. Inset: the plot is identical with that within Fig. 3

protein admixtures in the LDH preparations were followed via FPLC analyses (Fig. 1). Catalytically active LDH was identified within 11·5–13·5 ml (Fig 1, peak No. 1 from the left) whereby the protein concentration and LDH activity were found to be concomitant

The effect of protein admixtures on the elution behaviour is documented on chromatograms of both LDH preparations using CB-cellulose II as dye-ligand column (Figs 3 and 4). The double peaks in the elution profiles of crude LDH (Fig. 3) were ascribed to partial separation of LDH isoenzymes. Performing zonal elution of crude LDH from CB-cellulose III and IV (substituted to higher degree of substitution) similar profiles of chromatograms were found. On the contrary, using CB-cellulose column with a lower concentration of the immobilized dye (I), the elution profiles have been projected into single peaks similarly as in the previous work[3]. Simple elution peaks (resembling to the latter one) have been observed in the case when the purified LDH was applied onto CB-cellulose II (Fig. 4) and I. For all combinations of both variables (presented herein) the reciprocal plot of elution volume of LDH vs concentration of the mobile ligand (CB-dextran T 10) (Figs 3 and 4, inset) was linear. Consequently, the values of the respective dissociation constants could be calculated according to the equation valid for a monovalent interaction (Table II). The linearity was limited by upper concentration of CB-dextran T 10 (80–100 μmol CB l^{-1}).

As concluded in the previous work[3], the nature of interactions between LDH and the dye-ligand immobilized on bead cellulose was biospecific, since inclusion of a natural ligand (NADH) brought about a competitive elution of LDH. In con-

TABLE II

Review of dissociation constants K_{M-L} and K_{I-L}. Columns 1 and 2 denote preparations of LDH No. 1 (crude) and No. 2 (purified)

CB-cellulose	K_{M-L} μmol l^{-1}		K_{I-L} μmol l^{-1}		Correlation coefficient	
	1	2	1	2	1	2
I	9·1	3·1	1·6	1·2	0·992	0·998
II	4·4	2·9	1·55	1·9	0·998	0·994
	6·4		1·7		0·998	
III	8·1		1·9		0·986	
	12·2		2·0		0·975	
IV	20·0		3·15		0·962	
	8·0		1·0		0·988	

comitance the values of K_{I-L} were found to be lower than those of K_{M-L}. This has been interpreted in terms of additional binding interaction due to the dye-ligand in immobilized form.

In the present study the differences in K_{I-L} and K_{M-L} values have been confirmed. These differences may serve for estimation of the contribution of nonspecific interaction of the matrix to the total interaction between dye-ligand and the enzyme. This contribution (reflected in practically constant values of K_{I-L}) remained unchanged in the studied concentration range of the immobilized dye and in the presence of other protein admixtures.

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