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INTERACTION OF LACTATE DEHYDROGENASE WITH STRUCTURALLY RELATED TRIAZINE DYES USING AFFINITY PARTITIONING AND AFFINITY CHROMATOGRAPHY[a]

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SUMMARY

Affinity partitioning in aqueous two-phase systems consisting of dextran and dye-liganded polyethylene glycol was employed to study the interaction of lactate dehydrogenase (LDH) from rabbit muscle (E.C. 1.1.1.27) with Procion Red HE-3B and four structurally related derivatives of this dye in order to follow the significance of the terminal rings of Procion Red HE-3B for the strengh of interaction. The study revealed that the arrangement of the two 1-amino-8-naphthol-3,6-disulphonic acid rings seems to be a prerequisite for the interaction of azonaphthol dyes with LDH.

The negatively charged sulfonic acid group at the terminal rings of Procion Red HE-3B enhances the affinity of the ligand for LDH significantly. The removal of this sulphonic acid group or splitting off the complete terminal rings decreases the affinity to LDH and improves the competitive effect of NAD^+. The results of affinity partitioning are compared with those of affinity chromatography and kinetic data. The usefulness and the choice of parameters of affinity partitioning as an analytical tool to predict the chromatographic behaviour of dye ligands are discussed.

INTRODUCTION

Particular emphasis has been placed on the study of the interaction of reactive dyes, especially Cibacron Blue F3G-A, with lactate dehydrogenase (LDH) (E.C.

[a] Dedicated to Professor Dr. E. Hofmann, Director of the Institute of Biochemistry of the Karl-Marx University Leipzig, on the occasion of his 60th birthday.

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1.1.1.27) as a model enzyme, because this protein contains a single dinucleotide fold per subunit binding one dinucleotide non-cooperatively with high affinity[1]. Several groups employed kinetic studies[2,3], difference spectoscopy, equilibrium dialysis[1] and analytical affinity chromatography[4] to analyse the mechanism of triazine dye–LDH interaction.

In the last decade, dye affinity partitioning in aqueous two-phase systems has been utilized for studying dye–protein interactions in more detail[5–7]. The large amount of water (70–90%) and the low interfacial tension in aqueous two-phase systems provide an excellent environment for dissolving biological molecules such as enzymes and other proteins and for preserving their structure and activity. The high sensitivity in the recognition of any ligand–protein interaction and the simplicity of performing this procedure also favour this method.

For the characterization of the behaviour of enzymes in affinity partitioning, the parameter $\Delta \log K$, *i.e.*, the difference in the logarithms of the partition coefficients in the systems with and without a ligand, is usually determined. From the change in $\Delta \log K$ as a function of the ligand concentration, two parameters, the relative affinity of the ligand to the protein ($0.5\ \Delta \log K_{max}$) and the maximum extraction power ($\Delta \log K_{max}$) are obtained[8]. By studying the influence of effectors on the partition behaviour of the respective enzyme, information on the specificity and on the mode of interaction of the ligand–protein binding can be deduced.

The affinity partitioning of LDH in aqueous two-phase systems with different reactive dyes, especially with Cibacron Blue F3G-A coupled to polyethylene glycol (PEG), was studied by Kopperschläger *et al.*[5] and Johansson and Joelsson[9]; the latter applied Procion Yellow HE-3G as a dye ligand for purifying the enzyme from porcine muscle extract.

In this work, affinity partitioning was employed to study the binding behaviour of LDH for Procion Red HE-3B. Although this dye was found to interact preferentially with $NADP^+$-dependent enzymes[10], kinetic experiments gave evidence of a high affinity for LDH from rabbit muscle[11]. Particularly the effect of structural variations of Procion Red HE-3B on the affinity and specificity of the ligand–LDH interaction was analysed in more detail by affinity partitioning and the results were compared with those of affinity chromatography.

EXPERIMENTAL

Materials

Lactate dehydrogenase (E.C. 1.1.1.27) from rabbit muscle, NAD^+ and NADH were obtained from Sigma (St. Louis, MO, U.S.A.), polyethylene glycol 6000 (PEG, M_r = 6000–7500) and dextran 500 (M_r = 350 000–550 000) from Serva (Heidelberg, F.R.G.), Procion Red HE-3B, Red H-3B and Yellow HE-3G from ICI Organics Division (Blackley, U.K.) and Cibacron Blue F3G-A from Ciba Geigy (Basle, Switzerland). Vilmax Dye I and II were a generous gift from Dr. Mazza, Vilmax (Buenos Aires, Argentina). All other biochemicals were of analytical-reagent grade.

Preparation of the immobilized dye derivatives

Dye–PEG. The triazine dyes were covalently bound to PEG 6000 in aqueous alkaline solution and the dye–PEG derivatives were purified by extraction with

chloroform and ion-exchange chromatography on DEAE-cellulose (Serva) according to Johansson[6]. The purity of the conjugates was analysed by thin-layer chromatography on silica gel G 60 plates (Merck, Darmstadt, F.R.G.) with 1-butanol–2-propanol–ethyl acetate–water (20:35:10:35) as the eluent.

For the hydrogenation of the azo bonds of Procion Red HE-3B, coupled to PEG 6000, to the corresponding hydrazino analogue Red HE-3B(M1), 200 mg of the dye–PEG derivative were dissolved in 3 ml of 1% (w/v) sodium hydrogencarbonate and 50 mg of sodium borohydride were added slowly with stirring at room temperature within 30 min.

For the cleavage of the azo linkages yielding the dye Red HE-3B(M2), 200 mg of Procion Red HE-3B-PEG were dissolved in 3 ml of 1% (w/v) sodium carbonate and 100 mg of sodium dithionite were added in small portions with stirring within 6 h at 40°C. The reaction was followed by recording the decrease in the absorbance of Procion Red HE-3B at 350 nm and by the detection of the splitting product after thin-layer chromatography on silica gel plates with 2-aminobenzenesulphonic acid as reference with 1-butanol–2-propanol–ethyl acetate–water (20:35:10:35) as the eluent and 4-(dimethylamino)benzaldehyde (DMBA) as the staining substance.

The modified dye–PEG was purified by extraction with chloroform and ion-exchange chromatography as decribed above. The purities of the yellow-orange Procion Red HE-3B-PEG derivatives [(Red HE-3B(M1) and Red HE-3B(M2)] were analysed by thin-layer chromatography using UV detection and staining of the thin-layer plates with DMBA.

Dye–Sepharose 4B. Procion Red HE-3B, Vilmax Dye I and II and Cibacron Blue F3G-A were coupled to Sepharose 4B (Pharmacia, Uppsala, Sweden) as described by Hughes *et al.*[12]. The ligand concentration was determined by acid hydrolysis according to Clonis *et al.*[13] using the following molar absorption coefficients (l mol^{-1} cm^{-1}): Procion Red HE-3B (530 nm; 30 000), Cibacron Blue F3G-A (610 nm; 13 600), Vilmax Dye I (514 nm; 38 810), Vilmax Dye II (574 nm; 6334), Procion Yellow HE-3G (400 nm; 35 600) and Procion Red H-3B (530 nm; 18 000).

Procion Red HE-3B–Sepharose 4B was chemically modified according to Clonis[14]. The initial ligand concentration of the Procion Red HE-3B–Sepharose was not changed after chemical modification.

Enzyme assay

The activity of the LDH was assayed at 25°C as described by Bergmeyer[15] in 50 m*M* sodium phosphate buffer (pH 7.5). One unit of activity is defined as the amount of the enzyme that catalyses the conversion of 1 μmol of substrate per minute 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 (0.5 *M*) (pH 7.5) and NAD^+ (20 m*M*). The polymer concentrations are given as a percentage of the total system. The concentrations of the dyes in the systems were determined spectrophotometrically using the molar absorption coefficients as given above. A 4-g amount of a two-phase system containing about 10 units of LDH were brought to 25°C, equilibrated by gently mixing and centrifuged at 1500 *g* for 5 min. Samples were withdrawn from the phases and diluted with test buffer. Inhibition of the enzyme by the dye–PEG in the assay mixture was excluded by sufficient 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. The term $\Delta \log K$ was obtained from the difference in the $\log K$ of the system containing dye–PEG and that of a reference without the ligand.

Affinity chromatography

The chromatographic experiments were performed at 25°C. Disposable columns (30 × 8 mm I.D.) from Bio-Rad Labs. (France) containing a 0.6-ml bed volume of triazine-liganded Sepharose 4B were equilibrated using 100 mM sodium phosphate buffer (pH 7.5). The dialysed enzyme solution was applied continuously to the column until the activity emerging from the column matched the activity applied on the top. The unbound protein was removed by washing with equilibration buffer at a flow-rate of 35 ml/h. The binding capacity of the dye–Sepharose was calculated from the difference in the total loaded and the unbound enzyme activities. The bound enzyme was eluted specifically by 2 mM NAD^+ dissolved in the equilibration buffer. The enzyme activity still retained on the column was finally desorbed by 3 M sodium chloride solution to determine the overall recovery of this stop. Fractions of 4 ml were collected and assayed for enzyme activity.

RESULTS

Affinity partitioning

Fig. 1 shows the partition of LDH in aqueous two-phase systems consisting of dextran 500 and PEG 6000 as a function of the polymer concentrations and the volume ratio of the phases. The effect of affinity partitioning can most advantageously be studied if the enzyme is mainly concentrated in one of the polymer phases before introducing the ligand polymer of the opposite phase. As shown in Fig. 1, this is achieved for LDH in a system containing 9% (w/w) dextran and 6% (w/w) PEG.

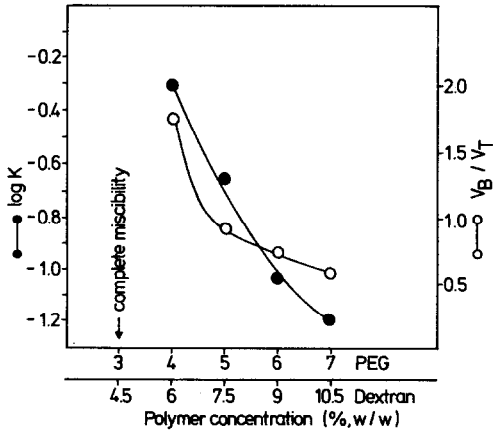

Fig. 1. Partitioning of lactate dehydrogenase in aqueous two-phase systems as a function of the concentration of the polymers. The systems (4 g) were composed of dextran 500, PEG 6000, 50 mM sodium phosphate buffer (pH 7.5) and 10 units of the enzyme. The systems were equilibrated at 25°C. V_B = volume of the bottom phase; V_T = volume of the top phase.

Under these condition, 90% of the total enzyme activity is concentrated in the dextran-rich bottom phase.

The affinity partitioning behaviour of LDH was studied in the presence of various dye ligands structurally related to Procion Red HE-3B covalently coupled to PEG 6000. The formulae of these dyes are shown in Fig. 2. Procion Red HE-3B, Red HE-3B(M1), Red HE-3B(M2) and Vilmax Dye I and II consist of two symmetrically arranged 1-amino-8-naphthol-3,6-disulphonic acid rings as the main structural element. Procion Red HE-3B can be regarded as the half molecule of Red HE-3B. With the exception of Red HE-3B(M1) and Red HE-3B(M2), all ligands are azonaphthol dyes with variations of the substituent (R).

By increasing the dye concentration in the two-phase systems an increase in $\Delta \log K$ was determined as shown by the hyperbolic curves in Fig. 3. Only with Procion Red HE-3B was the change in $\Delta \log K$ significantly less.

From the double reciprocal plots of the curves, the maximum extraction power ($\Delta \log K_{max}$) and the relative affinity of the dye ligand (0.5 $\Delta \log K_{max}$) were calculated (Table I). The highest value of $\Delta \log K_{max}$ was obtained with Vilmax Dye I and Red HE-3B(M2). Red HE-3B(M1) and Vilmax Dye II provided lower but comparable values. Procion Red HE-3B gave the lowest $\Delta \log K_{max}$ but showed a stronger relative affinity to LDH which is similar to Vilmax Dye II. The other dyes exhibited a lower

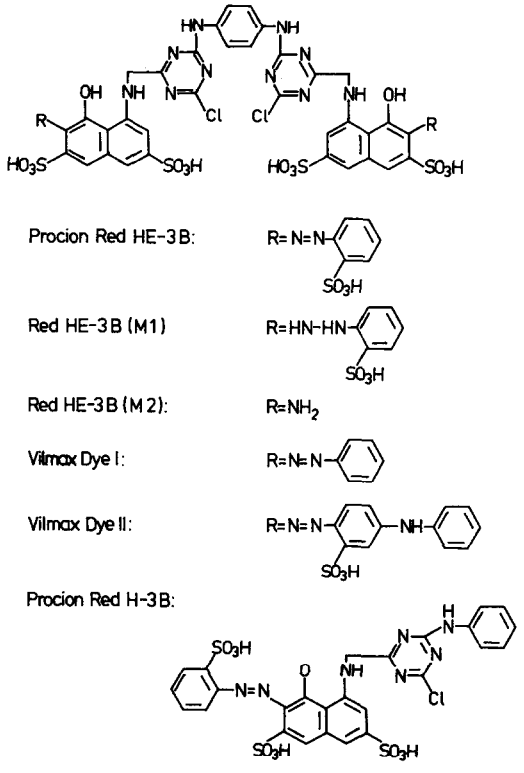

Fig. 2. Structure of Procion Red HE-3B derivatives.

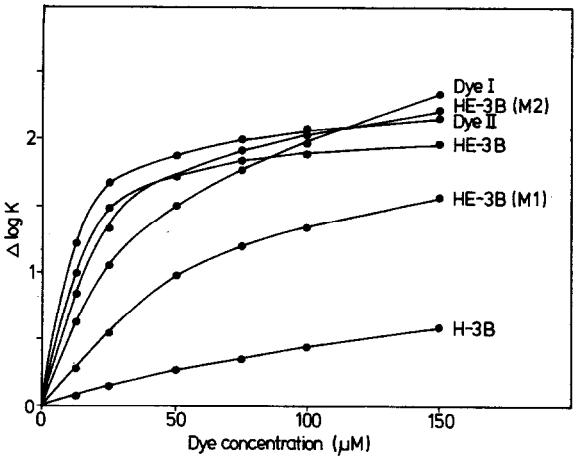

Fig. 3. Affinity partitioning of lactate dehydrogenase as a function of the concentration of various dye–PEG derivatives. The systems (4 g) contained 9% (w/w) dextran 500, 6% (w/w) PEG 6000 (partially replaced by dye–PEG), 50 m*M* sodium phosphate buffer (pH 7.5) and 10 units of the enzyme. The systems were equilibrated at 25°C.

relative affinity in the order Red HE-3B(M2) > Vilmax Dye I > Red HE-3B(M1).

For comparison, Table I also contains the $\Delta\log K_{max}$ values and the relative affinities of Cibacron Blue F3G-A and Procion Yellow HE-3G, two well known affinity ligands of LDH. Both dyes gave a high $\Delta\log K_{max}$ similar to the other dyes studied, but showed significant differences in their affinity to LDH. Whereas the 0.5 $\Delta\log K_{max}$ value of Procion Yellow HE-3G is similar to those of Procion Red HE-3B and Vilmax Dye II, the affinity of Cibacron Blue F3G-A is comparable to that of Vilmax Dye I.

In order to analyse the enzyme–dye interaction with respect to its specificity to

TABLE I

AFFINITY PARTITIONING OF LACTATE DEHYDROGENASE EXPRESSED BY THE MAXIMAL EXTRACTION POWER ($\Delta\log K_{max}$) AND THE RELATIVE AFFINITY (0.5 $\Delta\log K_{max}$) USING DIFFERENT DYE–PEG DERIVATIVES

The systems (4 g) contained 9% (w/w) dextran 500, 6% (w/w) PEG 6000, 50 m*M* sodium phosphate buffer (pH 7.5) and 10 units of the enzyme. The systems were equilibrated at 25°C. The data were calculated from double reciprocal plots of $\Delta\log K$ as a function of the dye–PEG concentration (see Fig. 3). The regression coefficients were between 0.99 and 1.00.

Dye–PEG	$\Delta\log K_{max}$	*Dye–PEG (μM) yielding* $0.5\ \Delta\log K_{max}$
Procion Red HE-3B	2.08	9.5
Red HE-3B(M1)	2.27	67.1
Red HE-3B(M2)	2.53	23.5
Vilmax Dye I	2.63	36.4
Vilmax Dye II	2.32	10.4
Cibacron Blue F3G-A	2.56	44.4
Procion Yellow HE-3G	2.63	7.2

the dinucleotide-binding domain, the effect of NAD^+ on the affinity partitioning of LDH was studied. The coenzyme NAD^+ partitions in the aqueous two-phase system used with a partition coefficient, $K = 0.85$, that is independent of the presence or absence of dye ligands.

Fig. 4 demonstrates that $\Delta \log K$ decreased significantly with increasing concentrations of NAD^+ in the systems. However, in none of the experiments was this parameter completely abolished. In Table II the effect of NAD^+ on the affinity partitioning of LDH in the presence of 50 μM dye–PEG is listed. The data for Cibacron Blue F3G-A and Procion Yellow HE-3G are included as references. At a final concentration of 2 mM NAD^+ in the systems, the differences in the residual $\Delta \log K$ values are relatively small. By comparing the concentrations of NAD^+ providing the half of the initial $\Delta \log K$, differences between the dye ligands become obvious.

NAD^+ competes most effectively with Vilmax Dye I and Red HE-3B(M1). Up to 4-fold higher concentrations of NAD^+ were necessary, leading to the same reduction of $\Delta \log K$ in the presence of Procion Red HE-3B, Red HE-3B(M2), Vilmax Dye II and Procion Yellow HE-3G. The effect of NAD^+ in competing with the Cibacron Blue F3G-A–LDH binding was minor, as with Red HE-3B(M1) and Vilmax Dye I, but significantly stronger than that with the other dye ligands.

Affinity chromatography

The results of the affinity chromatography of LDH on dye-liganded Sepharose

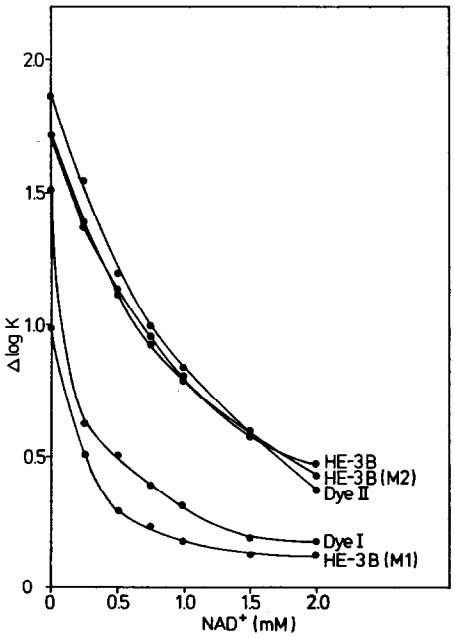

Fig. 4. Affinity partitioning of lactate dehydrogenase as a function of the amount of NAD^+ added to the system. The systems (4 g) contained 9% (w/w) dextran 500, 6% (w/w) PEG 6000 including 50 μM dye–PEG, 50 mM sodium phosphate buffer (pH 7.5) and 10 units of enzyme. The systems were equilibrated at 25°C.

TABLE II

INFLUENCE OF NAD^+ ON THE AFFINITY PARTITIONING OF LDH EXPRESSED AS THE CHANGE IN $\Delta \log K$

The systems (4 g) contained 9% (w/w) dextran 500, 6% (w/w) PEG 6000 including 50 μM dye–ligand, 50 mM sodium phosphate buffer (pH 7.5) and 10 units of LDH. The systems were equilibrated at 25°C. $\Delta \log K$ in the absence of NAD^+ was taken as 100%.

Dye–PEG	*NAD^+ (mM) yielding a reduction of 50% in $\Delta \log K$*	*Residual $\Delta \log K$ in the presence of 2 mM NAD^+ (%)*
Procion Red HE-3B	0.93	28
Red HE-3B(M1)	0.28	15
Red HE-3B(M2)	0.95	25
Vilmax Dye I	0.22	12
Vilmax Dye II	0.90	20
Cibacron Blue F3G-A	0.59	21
Procion Yellow HE-3G	0.91	32

4B are summarized in Table III. Although dye–Sepharoses were prepared by following the same procedure, different degrees of ligand substitution were obtained. Therefore, it was necessary to calculate the binding capacity of the dye–Sepharoses per unit of ligand concentration. The highest binding capacity was achieved with Procion Red HE-3B–Sepharose 4B. The binding capacity of Cibacron Blue F3G-A–Sepharose is comparable to that of the Vilmax Dye I- and Vilmax Dye II-substituted Sepharoses, but all three revealed a minor binding capacity in comparison with Procion Red HE-3B–Sepharose. The dye–Sepharoses liganded with Red HE-3B(M1) and Red HE-3B(M2) showed distinctly lower binding capacities.

By comparing the amount of enzyme activity that was eluted with 2 mM NAD^+ added to the buffer, differences between the dye–Sepharoses were discernible. A sufficient yield of activity was found using Red HE-3B(M1)- and Vilmax Dye I-liganded Sepharose. Less than 50% of the bound enzyme was eluted under the same

TABLE III

AFFINITY CHROMATOGRAPHY OF LACTATE DEHYDROGENASE WITH DYE-LIGANDED SEPHAROSE 4B

The experiments were performed at 25°C in columns (30 × 8 mm I.D.) equilibrated with 100 mM sodium phosphate buffer (pH 7.5) and with a flow-rate of 35 ml/h. The overall recovery was calculated from the difference of the bound enzyme activity and the activities eluted by successive washing with 2 mM NAD^+ and 3 M NaCl.

Dye–Sepharose	*Ligand concentration (μmol dye/g dried gel)*	*Binding capacity*		*Activity eluted with 2 mM NAD^+ (%)*	*Overall recovery (%)*
		U/ml gel	*U/μmol dye*		
Procion Red HE-3B	9.3	2408	8841	41	93
Red HE-3B (M1)	9.3	140	514	70	100
Red HE-3B (M2)	9.3	130	477	48	65
Vilmax Dye 1	2.0	72	1224	65	91
Vilmax Dye 11	7.7	261	1152	26	53
Cibacron Blue F3G-A	6.0	215	1218	49	93

conditions from the Procion Red HE-3B–, Red HE-3B(M2)–, Vilmax Dye II– and Cibacron Blue F3G-A–Sepharose, respectively. The overall recovery of the enzyme determined by subsequent washing off the column with 3 *M* sodium cloride solution was between 90% and 100%, with the exception of Red HE-3B(M2) and Vilmax Dye II (65% and 53%, respectively). Procion Red HE-3B immobilized to Sepharose is not able to bind substancial amounts of LDH (data not shown).

DISCUSSION

This study has shown that minute structural modifications of Procion Red HE-3B cause significant changes in the dye–LDH interactions, which are clearly recognized by means of affinity partitioning and affinity chromatography. Both approaches indicate that Procion Red HE-3B has a higher affinity and immobilized on Sepharose 4B also a higher binding capacity for LDH from rabbit muscle than Cibacron Blue F3G-A, the most characterized pseudo-ligand of the dinucleotide-binding domain of dehydrogenases[16]. The arrangement of two 1-amino-8-naphthol-3,6-disulphonic acid rings within the dye ligands seems to be a prerequisite for the azonaphthol class of dyes to bind LDH, as Procion Red HE-3B having only one 1-amino-8-naphthol-3,6-disulphonic acid ring shows a low affinity to the enzyme in comparison with all other dyes (for structures see Fig. 2). The addition of the anilino group to the terminal rings of Procion Red HE-3B giving Vilmax Dye II did not change its affinity to the enzyme significantly. However, the loss of the negatively charged sulphonic acid groups from both of the terminal rings as occurs in Vilmax Dye I weakens the affinity to LDH.

The differences in the affinity of Procion Red HE-3B and Vilmax Dye I and II for LDH from rabbit muscle as deduced by affinity partitioning are qualitatively consistent with kinetic parameters such as the dissociation constant of the dye–enzyme complex and the inhibition constant obtained with the free dyes under similar experimental conditions[11].

By comparing the structural differences between Procion Red HE-3B and Vilmax Dye I and II with the results of affinity partitioning, it appears that the negatively charged sulphonic acid group of the terminal 2-aminobenzene rings might be important for the binding of LDH. In order to verify this idea, Procion Red HE-3B was chemically modified. In fact, the removal of the sulphonated terminal rings [Red HE-3B(M2)] decreased the affinity for LDH. On the other hand, the drastic decrease in the affinity after hydrogenating the azo bridges [Red HE-3B(M1)] was unexpected, as the terminal negatively charged rings were retained. However, this result parallels the experimental data of Clonis[14], who found a lower binding strength of Procion Red HE-3B–Sepharose after the same chemical modification for porcine heart LDH.

The effect of hydrogenation of the azo bridges generates a more drastic structural change of the dye molecule than the formula in Fig. 2 indicates. As reported by Gordon and Gregory[17], the azo linkages of dyes such as Procion Red HE-3B form tautomeric structures producing intramolecular hydrogen bonds which are responsible for a restriction of the flexibility of the dye molecule. The hydrogenation of the azo group abolished this type of rigid, planar structure. However, protonation of the hydrazino groups generates positive charges in the dye molecule which compensate the negative charge of the terminal rings and might restrict the flexibility of the terminal

rings by forming intramolecular electrostatic bonds. Hence, finally the hydrogenation also generates a rigid dye molecule but without terminal negative charges, as occurs in Vilmax Dye I.

The competitive effect of NAD^+ as recognized by affinity partitioning and affinity chromatography are consistent. Dye ligands with a high affinity for LDH, such as Procion Red HE-3B, Procion Yellow HE-3G and Vilmax Dye II, compete with NAD^+ to a smaller extent than those showing a lower affinity (Vilmax Dye I and Cibacron Blue F3G-A). Also, the two derivatives of Procion Red HE-3B [Red HE-3B(M1) and Red HE-3B(M2)] corroborated this assertion.

The results indicate that the dinucleotide binding domain of the LDH from rabbit muscle is involved in the interaction of all dye ligands tested. However, the hydrophobicity of the terminal aromatic rings affects the affinity of the ligands for the NAD^+-binding site, as shown by comparing the effects of Procion Red HE-3B and Vilmax Dye I (Table I).

The low overall recovery, the high inactivation rate of LDH[11] and the lower competitive effect of NAD^+ indicate that structural changes as realized in Vilmax Dye II and Red HE-3B(M2) promote non-specific interactions. By comparing the maximum extraction power ($\Delta\log K_{max}$) with the binding capacity of the respective dye–Sepharoses (calculated per mole of immobilized dye ligand), no correlations were found. Dyes with a relatively low $\Delta\log K_{max}$ (*e.g.*, Procion Red HE-3B) showed a high binding capacity for LDH and *vice versa*. This result might be explained by the fact that the calculation of $\Delta\log K_{max}$ is based on the extrapolation for theoretically infinite high ligand concentrations. Moreover, the microenvironments of the ligand–enzyme binding in aqueous two-phase systems and on water-insoluble cross-linked agarose are different. In addition, with dyes covalently attached to a Sepharose matrix, only a small amount of the ligand is actually accessible to the enzyme, as shown by Liu *et al.*[4].

Along similar lines, Naumann *et al.*[18] found that PEG derivatives of Procion Green H-4G, Procion Orange MX-G, Procion Scarlet MX-G and Procion Yellow HE-3G, showing relative high $\Delta\log K$ values for LDH from porcine heart, are not able to bind the enzyme if they are coupled directly to cellulose. On the other hand, a direct correlation with the extraction power ($\Delta\log K$) and the binding capacity of the respective dye–Sepharoses can exist, as shown for alkaline phosphatase[19] and human serum pre-albumin[20].

The conclusion can be drawn that affinity partitioning is well suited to characterizing the dye–protein interaction in more depth. The method is able to reveal the influence of competing effectors on the dye–enzyme interaction. However, the maximum extraction power ($\Delta\log K_{max}$) cannot be used in a linear relation to predict the efficiency of the ligand in chromatographic systems. Although a low value of $\Delta\log K_{max}$ is related to the unsuitability of the ligand for affinity chromatography, a higher value does not reflect *per se* its usefulness as a ligand in affinity chromatography.

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