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APPLICATION OF AFFINITY PARTITIONING IN AN AQUEOUS TWO-PHASE SYSTEM TO THE INVESTIGATION OF TRIAZINE DYE–ENZYME INTERACTIONS

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SUMMARY

Affinity partitioning in an aqueous two-phase system by using triazine dye-substituted polyethylene glycol and dextran was applied in the investigation of the affinity of kinases, dehydrogenases and aminotransferases to twelve triazine dyes. From the alteration of the partition coefficient (K) of the enzymes in the two-phase system in the presence or absence of dye-liganded polyethylene glycol, expressed as $\Delta \log K$, quantitative data for the affinity of the enzymes to various triazine dyes were obtained. Optimal conditions for the purification of enzymes by applying dye–protein interaction were derived.

INTRODUCTION

The interaction of Cibacron Blue F3G-A and related triazine dyes with enzymes and other proteins, particularly with dehydrogenases and kinases, has attracted widespread interest in different areas of biochemistry and biotechnology[1–3]. Because the binding of most of the proteins is reversible and the immobilization of chlorotriazinyl dyes can be easily accomplished by a simple nucleophilic reaction, Cibacron Blue F3G-A and other dyes have been successfully applied as "pseudo-ligands" in affinity chromatographic procedures. A general theoretical concept of the chemical mechanism of binding has not been elaborated so far. The original idea of Stellwagen *et al.*[4] that Cibacron Blue F3G-A, by mimicing the natural ligands NAD^+ and ATP, is bound specifically to the nucleotide binding domain of dehydrogenases and kinases was found to be valid only in special cases[5].

Other triazine dyes, structurally unrelated to Cibacron Blue F3G-A, *e.g.*, Procion Red HE-3B, were also found to be capable of binding to certain dehydrogenases, kinases and other enzymes[6]. One reason for the preferred use of Cibacron Blue is its commercial availability as Blue Dextran, a Dextran 2000–Cibacron Blue F3G-A conjugate.

The procedures applied for the study of the interactions of proteins with the dyes involve kinetic, spectroscopic and binding techniques. Often they are difficult

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to interpret and yield contradictory results. In order to study the dye–enzyme interaction by a simple screening method, the technique of protein partitioning in an aqueous two-phase system, as developed by Albertsson[7], was applied. The two nonmiscible solutions contain dextran and polyethylene glycol in addition to triazine dye-substituted polyethylene glycol. The binding of a ligand with high affinity to one of the polymer is called affinity partitioning and has found successful application in the investigation of ligand–protein interactions[8–12].

EXPERIMENTAL

Materials

Phosphofructokinase (PFK) from baker's yeast (E.C. 2.7.1.11) and glycerol kinase (GK) from *Candida utilis* (E.C. 2.7.1.30) were prepared in our laboratory[13]. All other enzymes were obtained from Boehringer, Mannheim (G.F.R.): lactate dehydrogenase (LDH) (E.C. 1.1.1.27) and pyruvate kinase (PK) (2.7.1.40) from rabbit muscle, malate dehydrogenase (MDH) (E.C. 1.1.1.37), glutamate oxaloacetate transaminase (GOT) (E.C. 2.6.1.1) and glutamate pyruvate transaminase (GPT) (E.C. 2.6.1.2) from pig heart, glutamate dehydrogenase (GLDH) (E.C. 1.4.1.3) from beef liver and hexokinase (HK) (E.C. 2.7.1.1) from yeast.

Polyethylene glycol (M_r = 6000) (PEG 6000) was obtained from Ferak (Berlin West, G.F.R.) and Dextran T 500 (M_r = 500,000) from Pharmacia (Uppsala, Sweden). The dyes Cibacron Blue F3G-A and Cibacron Brilliant Blue BR-P were gifts from Ciba-Geigy (Basle, Switzerland), and the mono- and dichlorotriazine dyes of the Procion H and Procion M series were products of ICI Organics Division (Blackley, Manchester, Great Britain).

All other substrates, auxiliary enzymes and buffer substances were of analytical-reagent grade from Boehringer, VEB Laborchemie (Apolda, G.D.R.) and VEB Arzneimittelwerk (Dresden, G.D.R.). Of the dyes tested, only the structures of Cibacron Blue F3G-A, Cibacron Brilliant Blue BR-P, Procion Blue MX-3G, Procion Blue MX-R, Procion Red H-3B, Procion Red HE-3B and Procion Orange MX-G have been published[14]. Dye-substituted polyethylene glycol was prepared as described earlier[15]. The crude product was purified by ion-exchange chromatography on DEAE-cellulose, resulting in a monosubstituted PEG as the main product contaminated only by trace amounts of free dye[16].

Methods

The assay conditions for measuring the enzyme activities were described in ref. 17. The two-phase liquid–liquid system was prepared from aqueous solutions of PEG 6000 (20%, w/w), and Dextran T 500 (20%, w/w), a stock buffer of 0.5 *M* sodium phosphate, 50 m*M* 2-mercaptoethanol and 10 m*M* EDTA by weighing the respective amounts so that samples of 4 g contained 7% (w/w) Dextran, 5% (w/w) PEG in 50 m*M* sodium phosphate buffer (pH 7.0) and the respective amounts of the enzyme. For measuring the partition of hexokinase and glycerol kinase, 30 m*M* Tris–HCl buffer (pH 7.5) was used.

For the determination of the partition coefficients the tubes were cooled to 0°C, then about 1.5 units of each of the enzymes, dissolved in 50 µl of 50 m*M* sodium phosphate buffer (pH 7.0), were added, the mixture was shaken gently for about 15

TABLE I

PARTITION OF VARIOUS ENZYMES IN A TWO-PHASE SYSTEM COMPOSED OF 7% DEXTRAN T 500 (LOWER PHASE) AND 5% POLYETHYLENE GLYCOL (UPPER PHASE) EXPRESSED AS THE LOGARITHM OF THE PARTITION COEFFICIENT, *K*

Enzyme	*log K (n = 8)*
Pyruvate kinase	−1.2 ± 0.10
Phosphofructokinase	−1.2 ± 0.13
Lactate dehydrogenase	−0.73 ± 0.04
Malate dehydrogenase	−0.44 ± 0.07
Glutamate dehydrogenase	−0.48 ± 0.05
Glutamate oxaloacetate transaminase	−0.37 ± 0.03
Glutamate pyruvate transaminase	−0.36 ± 0.04
Glycerol kinase	−0.96 ± 0.02
Hexokinase	−0.55 ± 0.06

sec and then centrifuged at 2000 *g* for 2 min at 0°C. For assay of the enzyme activities appropriate volumes of each of the phase were carefully removed.

The partition coefficient, *K*, is defined as the ratio between the enzyme activities in the upper and lower phases when equal volumes of the two phases are compared. The recovery of activities of the enzymes in both phases was found to be more than 90% for all enzymes.

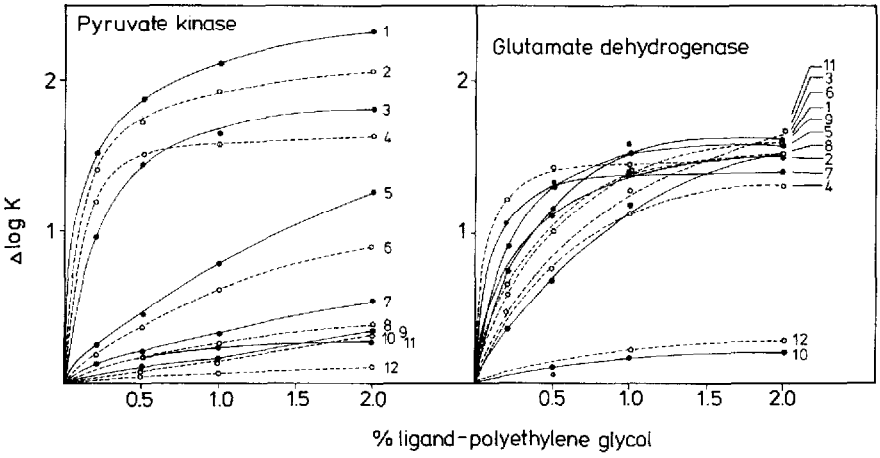

Fig. 1. Alteration of the partition coefficient of pyruvate kinase and glutamate dehydrogenase depending on concentrations of various dye–polyethylene glycol conjugates. The system (4 g) contains 5% (w/w) PEG 6000 with different portions of dye–PEG and 7% (w/w) Dextran T 500, 0.05 *M* sodium phosphate buffer (pH 7.0), 0.5 m*M* EDTA and 5 m*M* 2-mercaptoethanol. After cooling to 0°C about 1.5 units of each of the enzymes, dissolved in the same buffer, were added and the system was gently shaken for 15 sec. After separation of the phases samples from each of the two phases were removed for activity assay. Dyes: (1) Procion Navy HE-R; (2) Procion Green H-4G; (3) Procion Brown HE-G; (4) Procion Yellow HE-4R; (5) Procion Red HE-3B; (6) Procion Blue MX-3G; (7) Cibacron Brilliant Blue BR-P; (8) Procion Blue MX-R; (9) Cibacron Blue F3G-A; (10) Procion Red H-3B; (11) Procion Red HE-7B; (12) Procion Orange MX-G.

For characterization of the alteration of the partition of enzymes produced by dye-liganded PEG *versus* dye-free PEG the term $\Delta \log K$ was obtained from the difference in the log K values of a dye–PEG-containing system and a sample without dye–PEG.

RESULTS

The affinity of a given enzyme to diverse dye–PEG conjugates can most advantageously be studied when in the dye-free PEG system the enzyme is concentrated in the lower phase. This can be achieved in a system consisting of 7% Dextran T 500 and 5% PEG 6000 in 50 mM sodium phosphate (pH 7.0) (Table I).

After addition of the dye-substituted PEG in increasing portions at a constant level of total PEG expressed as percent of the total amount of PEG (*i.e.* 100%), those enzymes having affinity to the dye will be transferred to the dye–PEG-containing phase, *i.e.*, from the lower to the upper phase, which gives rise to a change in the log K value. Fig. 1 shows a representative experiment with two enzymes, pyruvate kinase and glutamate dehydrogenase, which exhibit different binding properties to a series

TABLE II

MAXIMUM ALTERATION OF THE PARTITION OF ENZYMES ($\Delta \log K_{max}$) BY VARIATION OF THE DYE-SUBSTITUTED POLYETHYLENE GLYCOL

Dye	$\Delta \log K_{max}$						
	>3	*2.5–3*	*2–2.5*	*1.5–2*	*1–1.5*	*0.5–1*	*<0.5*
Procion Navy HE-R	PFK		PK	GLDH, LDH	GPT, MDH	GOT, HK	GK
Procion Red HE-7B		PFK, GLDH		LDH	MDH	GPT	GOT, PK GK, HK
Procion Red HE-3B	PK	PFK		GLDH	LDH, MDH	GOT, GPT	GK, HK
Procion Red H-3B					PFK	MDH	GOT, GLDH GK, HK GPT, PK
Procion Orange MX-G					LDH		GOT, GLDH PK, GK, HK GPT, MDH
Procion Brown HE-G	PFK			GLDH, PK LDH	GOT, GPT MDH		HK, GK
Procion Yellow HE-4R			PFK	PK	GLDH, LDH	GOT, MDH	GK, HK
Procion Green H-4G	PFK		PK	GLDH, LDH		GOT, MDH GPT, HK	GK
Cibacron Blue F3G-A		PFK	GLDH	LDH	MDH, GPT	GOT	GK, HK
Cibacron Brilliant Blue BR-P		PFK		LDH	GLDH	GPT, MDH GOT, PK	GK, HK
Procion Blue MX-3G		PFK	PK	GLDH	MDH, LDH GPT	GOT	GK, HK
Procion Blue MX-R			PFK	GLDH	LDH	GOT, MDH GPT	PK, GK HK

of dye ligands. The curves describe the dependence of $\Delta \log K$ on the portion of dye–PEG in the system. In some instances the curves follow approximately a saturation function from which $\Delta \log K_{max}$ and the affinity of the dye to the enzyme have been calculated.

Table II summarizes the parameter $\Delta \log K_{max}$ for all of the enzymes tested. A maximum value of about 3 showed phosphofructokinase from yeast by using a series of selected dyes. This means that in such a system the distribution ratio of the enzyme changed 1000-fold when dye-free PEG was replaced by the dye-liganed PEG.

Most of the dehydrogenases studied exhibit a $\Delta \log K$ of about 1.5–2, decreasing from glutamate dehydrogenase to lactate dehydrogenase to malate dehydrogenase. The two transaminases undergo an alteration of the $\Delta \log K$ value between 0.5 and 1.5, whereas partitioning of glycerol kinase and hexokinase are hardly affected except for two dyes. Pyruvate kinase shows a surprising behaviour. Procion Red HE-3B, Procion Navy HE-R and Procion Green H-4G exhibit a high affinity to the enzyme ($\Delta \log K$ = 2–2.5). Cibacron Blue F3G-A and Procion Red HE-7B, however, were found to be unable to bind to the enzyme. The interaction of hexokinase with Procion Green H-4G, as reported in ref. 18, is weak and was found only in Tris–HCl buffer. A similar weak binding strength to the enzyme was shown by Procion Navy HE-R. Of all the dyes tested, Procion Red H-3B and Procion Orange MX-G are least applicable as affinity ligands for the enzymes investigated.

TABLE III

AFFINITY OF ENZYMES TO DIFFERENT TRIAZINE DYES EXPRESSED AS PERCENTAGE OF ΔLOG K_{max} AT 0.5% OF DYE–POLYETHYLENE GLYCOL CONCENTRATION

Dye	*PFK*	*PK*	*GK*	*HK*	*GLDH*	*LDH*	*MDH*	*GPT*	*GOT*
Procion Navy HE-R	97	79	47	50	81	97	88	70	60
Procion Red HE-7B	97	67	64	45	29	81	90	50	50
Procion Red HE-3B	94	14	73	40	77	77	90	56	65
Procion Red H-3B	38	20	60	60	50	7	40	25	50
Procion Orange MX-G	N.d.*	50	66	20	N.d.	26	33	50	50
Procion Brown HE-G	92	81	68	50	72	86	85	83	66
Procion Yellow HE-4R	96	94	55	40	62	83	69	79	61
Procion Green H-4G	N.d.	81	46	60	93	94	78	69	50
Cibacron Blue F3G-A	91	25	46	53	25	66	60	73	44
Cibacron Brilliant Blue BR-P	83	20	58	80	54	73	89	83	60
Procion Blue MX-3G	80	20	77	60	63	69	80	73	65
Procion Blue MX-R	72	45	95	40	67	77	63	66	50

* N.d. = Not determined.

For quantification of the affinity of the dye to a given enzyme, the portion of $\Delta \log K_{max}$ was defined as that obtained when in the upper phase 0.5% of the total PEG is replaced by the dye–PEG conjugate (Table III). Phosphofructokinase was found to exert a high affinity to most of the dyes, but the behaviour of other enzymes was different. According to Table II, Procion Orange MX-G and Procion Red H-3B have a low affinity to all enzymes studied. Consequently, the characterization of the dye–enzyme interaction for practical application requires a knowledge of the affinity of the dye to a given enzyme and the maximal extraction power of the dye–PEG phase.

The influence of various dyes on the partitioning of phosphofructokinase was studied in more detail. Owing to the well known affinity of this enzyme to Cibacron Blue F3G-A, it was interesting to compare it with other triazine dyes. Fig. 2 shows the results of partition experiments in which increasing concentrations of diverse dye–PEG conjugates were applied. In the douple reciprocal plots of $\Delta \log K$ *versus* the concentration of dye–PEG a set of straight lines were obtained. A maximal value of $\Delta \log K = 2.9$ was extrapolated for Cibacron Blue F3G-A, Procion Blue MX-G, Procion Red HE-3B and Cibacron Brilliant Blue BR-P, whereas Procion Navy HE-R produced $\Delta \log K = 3.6$ and Procion Red H-3B $\Delta \log K = 1.0$. From this graph

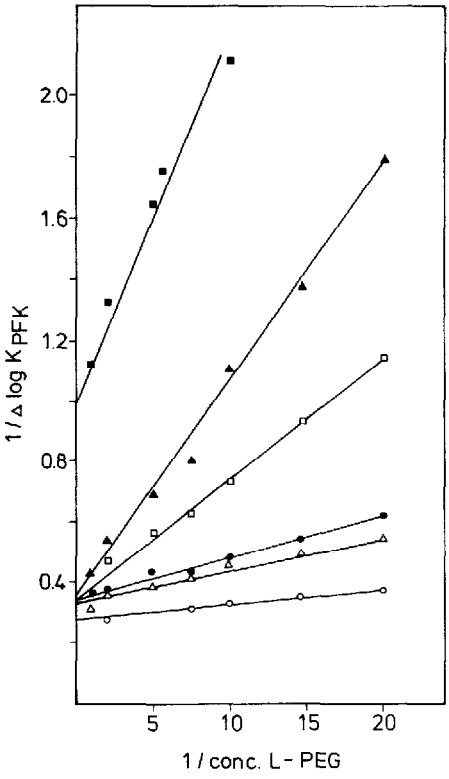

Fig. 2. Double reciprocal plots of $\Delta \log K$ for phosphofructokinase at increasing dye–polyethylene glycol concentrations. Experimental conditions as in Fig. 1. ○, Procion Navy HE-R; △, Procion Red HE-3B; ●, Cibacron Blue F3G-A; □, Cibacron Blue MX-3G; ▲, Cibacron Brilliant Blue BR-P; ■, Procion Red H-3B.

the relative affinities as calculated from the concentration of the dyes required for the half saturation point on the respective partition curve were evaluated. The latter was obtained from the intercepts of the lines with the abscissa. For Procion Navy HE-R a value of 0.017%, for Procion Red HE-3B 0.032%, for Cibacron Blue F3G-A 0.042%, for Procion Blue MX-G 0.12%, for Cibacron Brilliant Blue BR-P 0.2% and for Procion Red H-3B 0.12% of the dye–PEG calculated of the total amount of PEG (*i.e.* 100%) were found. These values express the variability of the dye affinities to phosphofructokinase. In addition, the usefulness of the affinity partitioning technique was tested for the evaluation of the influence of competing effectors on the binding of triazine dyes to enzymes, using lactate dehydrogenase and pyruvate kinase as model enzymes. In Fig. 3 the effects of ATP and MgATP as ligands for lactate dehydrogenase, respectively, at constant concentration of dye-PEG are shown.

For pyruvate kinase and ATP (Fig. 3A) in a Procion Navy H-ER–PEG-containing system, $\Delta \log K$ was only partially diminished. The MgATP complex shows a smaller effect than the free nucleotide. However, $\Delta \log K$ for lactate dehydrogenase in a system containing Cibacron Blue F3G-A–PEG becomes zero after adding 1 m*M* NAD^+ or 10 μM NAD^+ in the presence of sulphite, indicating a strong competition between the dinucleotide and the Cibacron Blue F3G-A molecule for the binding domain of the enzyme. Similar results were obtained with Cibacron Blue F3G-A and ATP in the case of phosphofructokinase from yeast[16].

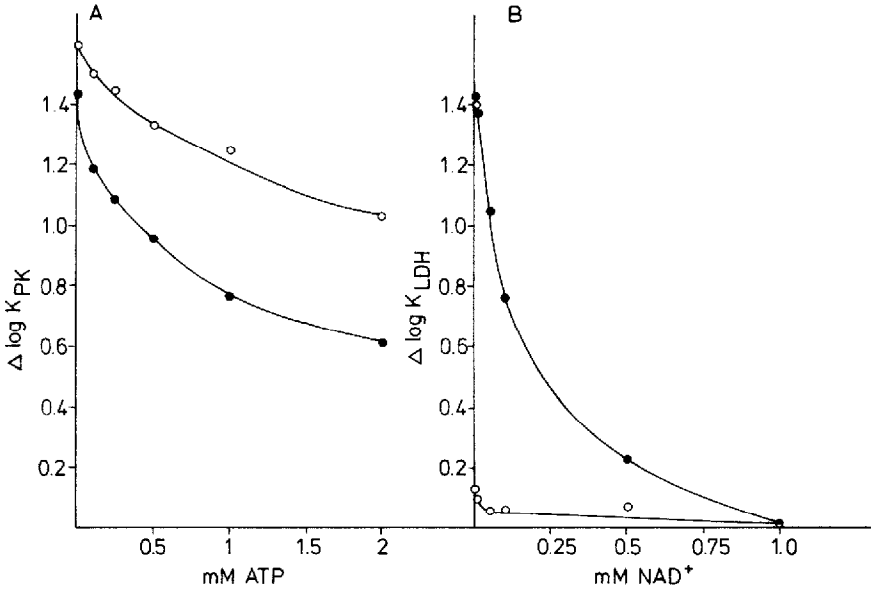

Fig. 3. Dependence of $\Delta \log K$ of pyruvate kinase and lactate dehydrogenase in a triazine dye–polyethylene glycol-containing system on the concentration of competing effectors. (A) System as in Fig. 1 with 0.2% of Procion Navy–PEG. Increasing amounts of ATP alone (●) or ATP + 2 m*M* $MgCl_2$ (○) were added to each sample and the log *K* value was determined as described under Experimental. (B) System as in Fig. 1 with 0.5% of Cibacron Blue F3G–PEG. Increasing amounts of NAD^+ (●) or NAD^+ + 2 m*M* sodium sulphite (○) were added before the enzyme was partitioned.

DISCUSSION

The interaction of proteins with dyes has attracted increasing interest for many purposes in analytical[19] and preparative[2] protein separations. For the selection of dyes suitable as affinity adsorbents for proteins, several screening techniques have been elaborated[20-22]. However, many of them are time consuming, and are strongly influenced by interfering factors, *e.g.*, the chemical nature of the resin and the degree of dye substitution[23].

In order to compare different triazine dyes as affinity ligands for enzymes and other proteins, the technique of affinity partitioning has been successfully applied. This simple and rapid method was found to be capable of giving the affinity of a dye–ligand to a protein and a giving comparable quantitative data. From the determination of the partition coefficient of an enzyme between two non-miscible phases in the presence and absence of a dye–ligand, the value of $\Delta \log K$ as a measure of the strength of the dye–enzyme interaction was found to be useful (Table II).

According to the theory of affinity partitioning, as developed by Flanagan and Barondes[8], the value of $\Delta \log K$ for a partitioning protein should be equal to the number of binding sites of the ligand per protein molecule multiplied by the logarithm of the partition coefficient of ligand–PEG concentration ($\log K_{\text{ligand-PEG}}$). This simple relationship can apparently not be applied to the binding of phosphofructokinase to Cibacron Blue F3G-A. For this the $\Delta \log K / \log K_{\text{ligand-PEG}}$ ratio is 1.5–2.6 (ref. 16), while the native enzyme in its octameric form is known to have sixteen binding sites for ATP[24] and probably also for Cibacron Blue F3G-A. Similar conclusions can also be drawn for other dyes bound to this enzyme (Table II).

From the dependence of the enzyme partitioning on the concentration of dye–PEG in the system, information about the affinity can be obtained, formally expressed as the dye–PEG concentration yielding a 50% value of the maximal $\Delta \log K$ for a given enzyme. Finally, the technique of affinity partitioning also permits the study of the influence of competing effectors on the dye–protein interactions. Taking lactate dehydrogenase as a model and NAD^+ and NAD^+/sulphite as competing effectors, the difference in the affinities of both for forming the enzyme NAD^+ and enzyme–NAD^+/sulphite complex[25], respectively, can also be recognized by the alteration of the partition coefficient.

The applicability of affinity partitioning to the large-scale purification of enzymes has been demonstrated by several groups[12,15,26]. From the results in this paper, optimal conditions for the purification of an enzyme by dye–ligand chromatography can be derived. This can be demonstrated by the following example. When the log K of an enzyme in the dye-free PEG system is -1 and $\Delta \log K_{\max}$ in the presence of the dye–ligand is about 3, it is possible to transfer about 99% of the desired enzyme from the lower phase into the upper phase in one step. In this instance the experimental conditions are simple and a high recoery is obtained. By combining various dye–PEG systems, enzymes with different $\Delta \log K$ values may be advantageously isolated by co-extraction in one step and separated from each other after changing the dye–PEG conjugate.

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