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Affinity partitioning of enzymes in aqueous two-phase systems containing dyes and their copper(II) complexes bound to poly(ethylene glycol)

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ABSTRACT

Affinity partitioning of lactate dehydrogenase from rabbit muscle was studied in aqueous two-phase systems containing many dyes bound to poly(ethylene glycol), and partitioning of lactate dehydrogenase and yeast glucose-6-phosphate dehydrogenase was studied with PEG derivatives containing Cu(II) complexes of the Red-Violet 2KT, Red-Brown 2KT and Claret 4CT. It was established that lactate dehydrogenase showed a higher affinity when it interacted with dyes without Cu^{2+} ions in their structure. Investigation of the partitioning of enzymes when sodium chloride, nucleotide ligands or chelating agents were introduced into two-phase systems showed that with the Cu(II) complex of Red-Brown 2KT the presence of Cu^{2+} ions in the dye resulted in partial loss of the specificity of its interaction with lactate dehydrogenase. In contrast to lactate dehydrogenase, the Cu(II) complex of this dye showed selectivity to glucose-6-phosphate dehydrogenase from yeast.

INTRODUCTION

Aqueous phase systems composed of poly(ethylene glycol) (PEG) and dextran or of PEG and salts have been successfully used for the extraction and purification of biomacromolecules [1–3]. Affinity partitioning of enzymes and proteins is a particular case of the partitioning technique. This technique is based on specific interaction of biomacromolecules with an affinity ligand covalently coupled to one of the phase system forming polymer. Consequently, a protein or an enzyme is preferentially concentrated in the phase containing the covalently attached ligand. Various fatty acids [4,5], alcohols [6], coenzymes [7] and triazine dyes [7–14] are useful as affinity ligands. Phase systems containing dyes as specific ligands are widely applied in the investigation of the affinity of biomacromolecules to triazine dyes [4,10,13,14].

Previously, we used the affinity partitioning technique for the comparative study of the interaction of the alcohol dehydrogenases (ADH) from yeast and horse liver with various dyes [15]. It was found that affinity partitioning of both ADH in two-phase systems containing dyes was most effective using Cu (II) complexes of the dyes Light Resistant Yellow 2KT, Red-Violet 2KT and Red-Brown 2KT (horse liver ADH). It was concluded [15] that the interaction of horse liver ADH with some dyes was also dependent on the presence of Cu^{2+} ions, and it was also assumed that the binding of enzyme to dyes might occur through the formation of the mixed Cu(II) complexes involving donor groups of the dyes and amino acid residues of the enzyme in a similar manner to yeast ADH. However, investigation of the interaction of yeast ADH and lactate dehydrogenase (LDH) from rabbit muscle with some dyes, *i.e.*, Yellow 2KT, Orange 5K and Light Resistant Yellow 2KT, in solution by differential

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and induced circular dichroism spectroscopy and chromatography on dye–adsorbents showed [16] that LDH interacted more specifically with dyes containing no metal ions.

In this work, the interaction of LDH from rabbit muscle with a range of dyes and dye–Cu(II) complexes was studied more thoroughly. Affinity partitioning of yeast glucose-6-phosphate dehydrogenase (G6PDH) in two-phase systems containing Cu (II) complexes of Red-Violet 2KT, Red-Brown 2KT and Claret 4CT attached to PEG was also studied for elucidation of the Cu^{2+} ions role.

EXPERIMENTAL

Chemicals

Lactate dehydrogenase from rabbit muscle and glucose-6-phosphate dehydrogenase from yeast were purchased from Serva (Heidelberg, Germany). Poly(ethylene glycol) PEG 6000 (mol. wt. 6000–7500) was obtained from Serva or Fluka (Basle, Switzerland) and dextran 60 000 (mol. wt. 60 000 ± 10 000 from the Factory of Clinical Preparations (Krasnojarsk, Russia). Tris, NADH, pyruvic acid, imidazole, adenine and glycylglycine were obtained from Sigma (St. Louis, MO, USA) and NADP and glucose-6-phosphoric acid disodium salt from Reanal (Budapest, Hungary). Reactive dyes were of local production, or obtained from Serva or Sigma [15]. All other chemicals were of analytical-reagent or high-purity grade.

Synthesis of dye–PEG derivatives

The dye-liganded PEGs were synthesized according to the method of Johansson and Joelsson [13] modified in such a manner that dye–PEGs were extracted supplementarily with chloroform before the chromatographic purification steps. After extraction, the dye–PEG derivatives were purified on columns of DEAE-cellulose DE-52 (Whatman, Maidstone, UK), and isolated by chloroform extraction. Cu^{2+} ions were removed from the PEG–Light Resistant Yellow 2KT,–Red-violet 2KT and –Red-Brown 2KT conjugates by washing these conjugates adsorbed on an anion exchanger (DEAE-cellulose or macroporous silica supports possessing anion-exchange groups) with 0.1 *M* EDTA solution. Dye–PEG derivatives were eluted with salts or acetone and finally the products were isolated by chloroform extraction [13,15]. Dye–PEG conjugates with the amount of dye as indicated earlier [15] were used.

Enzyme assays

The enzyme activities were determined spectrophotometrically at 340 nm at 30°C. Lactate dehydrogenase (E.C. 1.1.1.27) was determined according to Worthington [17] and glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) as described previously [18].

Two-phase systems

Two-phase systems (4 g) were prepared by weighing from stock solution of 20–50% (w/w) PEG, 20–30 (w/w) dextran and 0.1 *M* Tris–HCl buffer (pH 7.0) containing 5 m*M* EDTA for LDH and 0.2 *M* Tris–HCl buffer (pH 7.5) containing 5 m*M* Mg $(NO_3)_2 \cdot 6H_2O$, 10 m*M* EDTA, 5 m*M* 2-mercaptoethanol and 5% glycerine for G6PDH. A two-phase system composed of 6.5% (w/w) PEG 6000 and 10% (w/w) dextran in 10 m*M* Tris–HCl buffer (pH 7.0) containing 0.5 m*M* EDTA was used for LDH and a system composed of 7.0% (w/w) PEG 6000 and 10% (w/w) dextran in 20 m*M* Tris–HCl buffer (pH 7.5) containing 5 m*M* $Mg(NO_3)_2 \cdot 6H_2O$, 1.0 m*M* EDTA, 0.5 m*M* 2-mercaptoethanol and 0.5% glycerine for G6PDH. The amount of dye–PEG derivative is given as a percentage of the total mass of PEG present in the system or expressed as dye concentration (μM/kg) per kg of two-phase system.

Affinity partitioning of enzymes

After 2–20 units of LDH or 2–8 units of G6PDH had been introduced into the two-phase systems, the mixture was shaken gently for about 15 s, kept for 5 min and then centrifuged at 2000 *g* for about 2 min at 4°C to complete the phase separation. Samples of known volume were withdrawn from each phase and analysed for enzyme activity. The partition coefficient of the enzyme, *K*, defined as the ratio of enzyme concentration in the upper and lower phases, was calculated. The change in enzyme partition coefficients, $\Delta \log K$, was defined as the difference between the logarithmic partition coefficients of enzymes in the presence (K) and in the absence (K_0) of dye ($\Delta \log K = \log K - \log K_0$).

All the affinity partition curves of the enzymes

were linearized in double reciprocal plots of $\Delta\log K$ *vs.* dye concentration, treated by regression analysis and the due concentration which is required for the half-saturation point of the enzyme partition curve calculated according to Kopperschläger *et al.* [10]. The change in the enzyme partition coefficients when different selected agents were introduced into two-phase systems was expressed as a percentage of the initial value of $\Delta\log K$ in the presence of the dye and the absence of the agent. A decrease in $\Delta\log K$ by 100% was taken as $\Delta\log K = 0$.

RESULTS

Partitioning of LDH

The affinity of an enzyme to dye–PEG derivatives can advantageously be studied when the enzyme is concentrated in the lower phase of the dye–PEG-free two-phase system. Using a two-phase system consisting of 6.5% PEG 6000 and 10% dextran 60 000, the partition coefficient of LDH from rabbit muscle is 0.0088 ± 0.0008, *i.e.*, it exceeds 99% of the LDH introduced into the system in the bottom dextran-rich phase. Replacement of a portion of PEG with increasing portions of dye–PEG leads to changes in the enzyme logarithmic partition coefficients, rising to a maximum value $\Delta\log K_{max}$, which was regarded as a measure of the enzyme partitioning effect.

As seen from Table I, LDH is effectively transferred into the dye–PEG-containing upper phase when PEG derivatives with dyes from Procion Blue SP-3R ($\Delta\log K_{max} = 2.80$) to Claret 4CT–Cu(II) (1.93) were present in the system. Less extraction of the enzyme into the upper phase occurs in the presence of the PEG conjugates with Light Resistant Yellow 2KT–Cu(II) ($\Delta\log K_{max} = 1.40$), Red-Violet 2KT–Cu(II) (1.50) and Bright Yellow 2KT (0.88). The relative affinities of the enzyme to the dyes, calculated as the concentration required to yield $0.5\Delta\log K_{max}$, decrease in the order Procion Green H-4G (2.6 μM/kg of two-phase system), Orange 5K (35.0), Cibacron Blue F3G-A (47.0), Bright Yellow 53 (58.0) and Procion Blue MX-R (76.0). The affinity of other dyes was lower and the concentration of dyes yielding $0.5\Delta\log K_{max}$ was in the range 127–205 μM/kg.

It is noticeable that the LDH extraction power

TABLE I

INFLUENCE OF DIFFERENT PEG–DYE DERIVATIVES ON THE AFFINITY PARTITIONING OF RABBIT MUSCLE LACTATE DEHYDROGENASE

Two-phase system (4 g) composition: 6.5% (w/w) PEG 6000, 10% (w/w) dextran 60 000, 10 mM Tris–HCl buffer (pH 7.0), 0.5 mM EDTA.

Dye–PEG	$\Delta\log K_{max}$	Dye yielding $0.5\Delta\log K_{max}$ (μM/kg)
Light Resistant Yellow 2KT–Cu(II)	1.40 (1.10)[a]	205
Red-Violet 2KT–Cu(II)	1.50 (1.10)[a]	127
Procion Blue SP-3R	2.80	n.d.[b]
Cibacron Blue F3G-A	2.78	47
Procion Yellow H-3R	2.60	n.d.
Bright Red 6C	2.35	153
Procion Blue MX-R	2.40	76
Red-Brown 2KT–Cu(II)	2.64 (2.23)[a]	128
Red-Brown 2K	2.50	n.d.
Procion Green H-4G	2.65	2.6
Orange 5K	2.75	35
Bright Yellow 53	2.54	58
Claret 4CT–Cu(II)	1.93	201
Bright Yellow 2KT	0.88	n.d.

[a] Dye–PEG derivatives without Cu^{2+} ions.
[b] n.d. = Not determined.

($\Delta\log K_{max}$) and the relative affinity of dye to the enzyme with Cibacron Blue F3G-A (Table I) agree with the results obtained by Kirchberger *et al.* [19].

It may be concluded from the data in Table I that generally, LDH exhibits a higher affinity and is more effectively extracted into the upper phases containing dyes with no metal ions in their structure and interacts more weakly with dye–PEG derivatives containing coordinated Cu^{2+}ions. However, in the latter instance some difference in the interaction of the dye–Cu(II) complexes with the enzyme should be noted. For example, more effective extraction of LDH into the upper phase was observed with the Cu(II) complexes of Claret 4CT ($\Delta\log K_{max}$ = 1.93) and Red-Brown 2KT (2.64) in comparison with Cu(II) complexes of Light Resistant Yellow 2KT and Red-Violet 2KT ($\Delta\log K_{max}$ = 1.40–1.50).

A comparative study of the partitioning of LDH in the presence of the Cu(II) complexes of dye–PEG derivatives and their analogues after Cu^{2+}ions had been removed is summarized in Table II. It is obvious that the contribution of the Cu^{2+}ions is greater in the interaction of LDH with Red-Brown 2KT and Claret 4CT. Removal of the Cu^{2+}ions from these dyes resulted in a decrease in $\Delta\log K$ of LDH by 46% when equal concentrations of dye with no Cu^{2+} and dye–Cu(II) complex were participating in the system. For Light Resistant Yellow 2KT and Red-Violet 2KT the decrease in $\Delta\log K$ of the enzyme reached 29–31%. These data contrast with the results reported previously [15] by determiningg the role of Cu^{2+}ions in the interaction of dyes with yeast and horse liver ADH. It was found [15] that the affinity extraction of both ADH into the upper phases was greater with Cu(II) complexes of Light Resistant Yellow 2KT, Red-Violet 2KT (yeast ADH) and additionally Red-Brown 2KT (horse liver ADH) than partitioning in the presence of PEG–dyes containing no metal ions as cited in Table I. It was determined [15] that the removal of Cu^{2+}ions from PEG–Light Resistant Yellow 2KT–Cu(II) caused a decrease in $\Delta\log K$ of yeast ADH by 93%. For horse liver ADH and PEG conjugates with Light Resistant Yellow 2KT, Red-Violet 2KT and Red-Brown 2KT after the removal of Cu^{2+}ions from the latter the decrease in $\Delta\log K$ reached 77, 78, and 76%, respectively. Comparison of these findings clearly demonstrates that the contribution of Cu^{2+} is smaller in the formation of LDH complexes with the above-mentioned dyes than for yeast and horse liver ADH.

Partitioning of LDH in the presence of selected agents

The evaluation of the specificity of dyes to LDH, the character and the nature of the forces of the binding of dyes to the enzyme were studied by affinity partitioning of the enzyme in the presence of NaCl, NADH and chelating agents (imidazole, adenine and EDTA). The compounds were selected based on previously reported data [16] by studying the eluting capacity of various agents in yeast ADH affinity chromatography on an adsorbent with an immobilized Cu(II) complex of Light Resistant Yellow 2KT and the ability of the agents to participate in the formation of mixed Cu(II) complexes with

TABLE II

INFLUENCE OF Cu^{2+} IONS ON THE AFFINITY PARTITIONING OF LACTATE DEHYDROGENASE

System composition as in Table I.

Dye–PEG	Concentration of dye (μM/kg)	$\Delta\log K$		Decrease in $\Delta\log K$ in the presence of dye–PEG without Cu^{2+} (%)
		Cu^{2+}	–	
Light Resistant Yellow 2KT	400	1.36	0.96	29
Red-Violet 2KT	450	1.42	0.98	31
Claret 4CT	200	1.30	0.70	46
Red-Brown 2KT	350	2.60	1.40	46

this dye, and as a result their ability to displace the enzyme from the coordination sphere of the Cu^{2+} ions.

Table III shows that 1 m*M* NADH, as a specific agent of LDH, introduced in a PEG–dye system with no metal ions could reduce the value of $\Delta\log K$ by 85–105%. This leads to the assumption that this group of dyes might be expected to interact with enzyme specifically at the nucleotide ligand binding domain. A similar conclusions was made by studying the effect of structural variations of Procion Red HE-3B on the affinity and specificity of the ligand LDH interaction [19].

Addition of NaCl and an increase in its concentration in the phase systems up to 0.6 *M* caused a decrease in $\Delta\log K$ by 52–88% in the presence of the same group of PEG–dyes. This could indicate that electrostatic binding forces were involved and dominated over other forces in the formation of the dye-LDH complexes.

The effects of NADH and NaCl on the changes in $\Delta\log K$ for the studied PEG–dye–Cu(II) complexes differ greatly (Table III, Fig. 1). Low concentrations of NADH (1 m*M*) or NaCl (0.2 *M*) had resulted in the reduction of $\Delta\log K$ by 100 and 57–72%, respectively, in the cases of PEG–Light Resistant Yellow 2KT–Cu(II) and PEG–Red-Violet 2KT–Cu (II). Hence it may be concluded that Cu(II) complexes of both dyes behaved towards the enzyme similarly to a group of dyes containing no metal ions. However, the $\Delta\log K$-reducing capacity of the analogous concentration of NADH and NaCl by 26 and 5%, respectively, seemed insufficient in the case of PEG–Red-Brown 2KT–Cu(II). Such a change in $\Delta\log K$ in the latter instance correlates with the data in Table II. As indicated, the contribution of Cu(II) ions in the interaction between Red-Brown 2KT and LDH had been more important in comparison with Light Resistant Yellow 2KT and Red-Violet 2KT and, as a result, the low concentrations of NADH and NaCl were not capable of destroying the LDH–Red-Brown 2KT–Cu(II) complex.

Previously, we determined [15] that for the disruption of complexes formed by ADH and PEG–dyes with coordinated Cu^{2+} ions the concentration of selected agents added to two-phase systems should increase as the strength of the interaction of the dye–Cu(II) complexes with enzymes did. This is also valid for systems containing PEG–Red-Brown 2KT–Cu(II) in the presence of NADH. An increase in its concentration to 5 m*M* increased the $\Delta\log K$ reduction to 82%. However, an increase in the concentration of NaCl to 0.6 *M* had no appreciable effect on the change in $\Delta\log K$. The effect of chelat-

TABLE III

DEPENDENCE OF THE AFFINITY PARTITION EFFECT ($\Delta\log K$) OF RABBIT MUSCLE LACTATE DEHYDROGENASE ON THE CONCENTRATION OF NaCl AND NADH

System composition as in Table I.

Dye–PEG	Concentration of dye (μM/kg)	Decrease in $\Delta\log K$ (%)				
		NADH (m*M*)			NaCl (*M*)	
		0.5	1.0	5.0	0.2	0.6
Orange 5K	250	70	89	115	63	85
Procion Green H-4G	80	92	104	125	66	88
Procion Blue SP-3R	1%	95	96	105	29	52
Cibacron Blue F3G-A	200	58	92	114	61	85
Procion Blue MX-R	320	100	105	126	55	82
Procion Yellow H-3R	1%	88	102	117	n.d.	n.d.
Bright Red 6C	350	88	95	100	72	85
Bright Yellow 53	250	74	85	95	58	88
Light Resistant Yellow 2KT–Cu(II)	280	74	100	152	57	48
Red-Violet 2KT–Cu(II)	380	86	100	114	72	76
Red-Brown 2KT–Cu(II)	250	15	26	82	5	20
Red-Brown 2KT	250	108	108	114	79	85

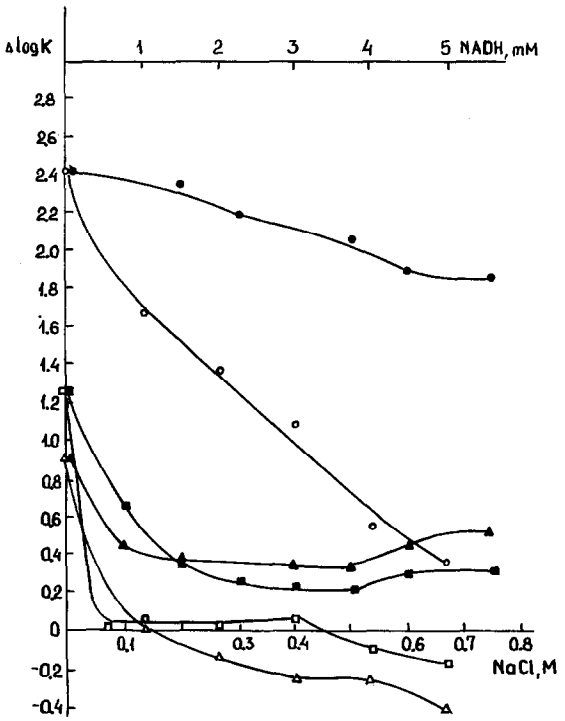

Fig. 1. Partitioning of lactate dehydrogenase in systems containing increasing concentrations of NADH and NaCl in the presence of dye–Cu(II) complexes. System composition as in Table I, dye–PEG concentrations as in Table III. Dye–PEG: ○, ● = Red-Brown 2KT–Cu(II); □, ■ = Red-Violet 2KT–Cu(II); △, ▲ = Light Resistant Yellow 2KT–Cu(II). Open symbols for systems containing NADH, closed symbols for NaCl.

ing agents on the affinity partitioning of LDH was studied in the presence of PEG conjugates with Light Resistant Yellow 2KT–Cu(II) and Red-Brown 2KT–Cu(II) as cases differing in the contribution of Cu^{2+}ions to the formation of dye–LDH complexes. Fig. 2 and Table IV show that of the chelating agents used, *i.e.*, EDTA, adenine and imidazole, the last is the most effective for the destruction of the LDH–PEG–Red-Brown 2KT–Cu(II) complex. For example, 0.5 m*M* imidazole caused a decrease in $\Delta\log K$ of 68%, whereas the same concentration of NADH gave only a 15% decrease. In contrast, the effect of 0.5 m*M* imidazole on the decrease in $\Delta\log K$ is smaller in the presence of PEG–Light Resistant Yellow 2KT–Cu(II), whereas 0.5 m*M* NADH reduced $\Delta\log K$ of LDH by 74% (Table III).

These data serve as a further confirmation that Cu^{2+}ions play a more important role in the interaction of Red-Brown 2KT–Cu(II) with LDH. It is worth noting that chelating agents in concentrations up to 5 m*M* reduced $\Delta\log K$ insignificantly (by 16–45%) when the PEG conjugate with Red-Brown 2KT after the removal of Cu^{2+}ions was used (Fig. 3), whereas 1.0 m*M* NADH or 0.2 *M* NaCl effectively destroyed this dye–LDH complex (Table III). According to these data, it is evident that Red-Brown 2KT becomes more specific to LDH after Cu^{2+}ions have been removed and could interact with enzymes similarly to the group of dyes containing no metal ions.

Partitioning of yeast glucose-6-phosphate dehydrogenase in the presence of selected agents

Partitioning data for the enzyme, in the presence of PEG conjugates with Cu(II) complexes of Red-Brown 2KT, Red-Violet 2KT and Claret 4CT when NaCl, NADP or chelating agents were introduced into the phase systems, are summarized in Table V. It is obvious that in all the cases studied the specific

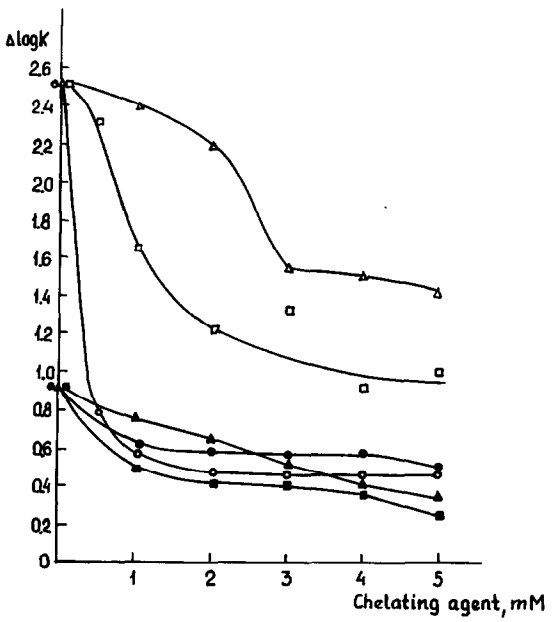

Fig. 2. Partitioning of lactate dehydrogenase in systems containing increasing concentrations of chelating agents in the presence of PEG–Light Resistant Yellow 2KT–Cu(II) and PEG–Red-Brown 2KT–Cu(II). System composition and dye–PEG concentrations as in Table III. ○ = Imidazole; □ = adenine; △ = EDTA; Open symbols for PEG–Red-Brown 2KT–Cu(II), closed symbols for PEG–Light Resistant Yellow 2KT–Cu(II).

TABLE IV

DEPENDENCE OF THE AFFINITY PARTITION EFFECT (Δlog K) OF RABBIT MUSCLE LACTATE DEHYDROGENASE ON THE CONCENTRATION OF CHELATING AGENTS

System composition and dye–PEG concentration as in Table III.

Agent	Concentration (mM)	Decrease in Δlog K (%)		
		Red-Brown 2KT–Cu(II)	Light Resistant Yellow 2KT–Cu(II)	Red-Brown 2KT
Imidazole	0.5	68	23	10
	5.0	81	44	16
Adenine	0.5	8	27	19
	5.0	63	74	36
EDTA	0.5	1	7	21
	5.0	43	63	45

agent NADP, at concentrations up to 1 mM, was capable of destroying the enzyme–dye complexes and this led to the assumption that the tested dye–Cu(II) complexes probably interacted with the enzyme at nucleotide ligand binding sites. The non-specific agent NaCl influenced the change in the enzyme partition coefficient by a different mode. At 0.2 M NaCl Δlog K of the enzyme was reduced by 46, 66 and 113%, respectively, in the cases of PEG conjugates with Red-Brown 2KT–Cu(II), Red-Violet 2KT–Cu(II) and Claret 4CT–Cu(II). A further increase in the concentration of NaCl (up to 0.4 M) resulted in decreases Δlog K by 87% and 107% with

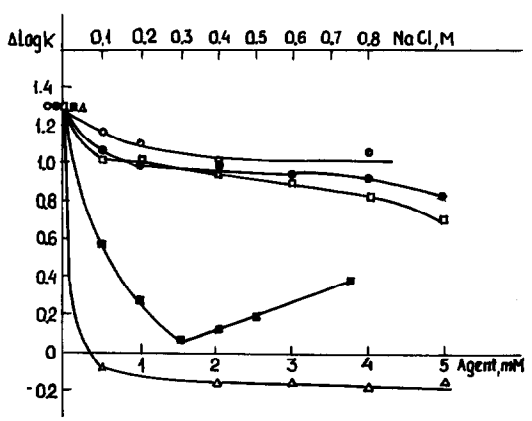

Fig. 3. Partitioning of lactate dehydrogenase in systems containing increasing concentrations of various agents in the presence of Red-Brown 2KT wihtout Cu^{2+}ions. Systems composition and dye–PEG concentration as in Table III. ○ = Imidazole; ● = adenine; □ = EDTA; ■ = NaCl; △ = NADH.

PEG–Red-Brown 2KT–CU(II) and Red-Violet 2KT–Cu(II), respectively. The different effects of the same concentration of NaCl on the decrease in Δlog K over the range of dye–Cu(II) complexes (from Red-Brown 2KT to Claret 4CT) could be attributed to the participation of a different part of the Cu^{2+} coordination bonds in the formation of the enzyme–dye complexes. This is confirmed by the enzyme partitioning in the presence of chelating agents such as imidazole and adenine. It is seen (Table V) that low concentrations of these agents decreased Δlog K most efficiently in the case of PEG–Red-Brown 2 KT–Cu(II). As 1 mM NADP also caused an effective decrease in Δlog K, it could be concluded that the Cu(II) complex of Red-Brown 2KT is more specific to yeast G6PDH, in contract to LDH from rabbit muscle. Probably, it will be able to participate in the formation of highly selective ternary complexes involving G6PDH, Cu^{2+}ions and dye similarly to the yeast ADH–Cu (II) complex of Light Resistant Yellow 2KT studied previously [29].

DISCUSSION

The technique of affinity partitioning of LDH from rabbit muscle [19–24] and G6PDH from yeast [21,25–27] in aqueous two-phase systems containing dye-liganded polymers has been applied to the screening of dyes with respect to their affinity to the enzymes. The possibilities of rapid purification of

TABLE V

DEPENDENCE OF THE AFFINITY PARTITION EFFECT ($\Delta\log K$) OF YEAST GLUCOSE-6-PHOSPHATE DEHYDROGENASE ON THE CONCENTRATION OF NaCl, NADP AND CHELATING AGENTS

Two-phase system (4 g) contained 7% (w/w) PEG 6000, 10% (w/w) dextran 60 000, 20 m*M* Tris–HCl buffer (pH 7.0), 5.0 m*M* $Mg(NO)_3 \cdot 6H_2O$, 1.0 m*M* EDTA, 0.5 m*M* 2-mercaptoethanol and 0.5% (v/v) glycerine.

Agent	Concentration (m*M*)	Decrease in $\Delta\log K$ (%)		
		Red-Brown 2KT–Cu(II) (357 μM/kg)	Claret 4CT–Cu(II) (425 μM/kg)	Red-Violet 2KT–Cu(II) (510 μM/kg)
NaCl	200	46	113	66
NADP	1.0	138	90	$\Delta\log K = 0$
Imidazole	1.0	77	20	20
	2.5	94	32	48
Adenine	1.0	61	14	26
	2.5	87	52	52

enzymes using affinity partitioning have also been demonstrated on medium [28] and large scales [23].

It was shown [20] that the dye Procion Yellow HE-3G interacted strongly with LDH from rabbit muscle, and various polymers as dye carriers were compared [22]. Finally, this dye was used as a conjugate with PEG for the large-scale affinity extraction of the enzyme using a PEG–Aquaphase PPT two-phase system [23]. Recently, Kirchberger and co-workers studied the binding behaviour of LDH [19] and its isoenzymes [24] to different triazine dyes covalently coupled to PEG, and showed a high relative affinity of Procion Red HE-3B to LDH and its isoenzymes. With the aid of Procion Yellow HE-3G coupled to various polymers, yeast G6PDH could be effectively extracted, as has been demonstrated [21,25,27].

Based on the data presented (Tables I and III), it may be concluded that some of the dyes studied in this work could also act as relatively high affinity ligands for LDH from rabbit muscle and probably other NAD(H)-dependent dehydrogenases. This is confirmed by the fact that adsorbents carrying Orange 5K and Bright Yellow 5Z have been used successfully as highly specific materials for the large-scale purification of some dehydrogenases, including LDH from rabbit muscle (data not shown).

Some of the dyes studied, Light Resistant Yellow 2KT, Red-Brown 2KT, Red-Violet 2KT and Claret 4CT, exsist as Cu(II) complexes in which the coordination of the Cu^{2+} ions involves an azo bond and two adjacent hydroxyl moieties of the dyes. The remaining coordination site could be occupied by appropriate protein donor groups. Therefore, these structures are useful as a means for the investigation of metal ion-promoted binding of proteins to dyes including also the technique of affinity partitioning [15].

Metal ion-dependent interactions between dyes and proteins are well documented [15,16,29–37]. It has been shown [29,30,33,34,38] that metal ions could enhance the binding of dyes to proteins by lowering the dissociation constant for the dye–enzyme interaction. The dye–enzyme complexes produced could be broken by selected competing effectors [16,30,31,35] or appropriate chelating agents [15,16,32,35,39]. Recently [37], metal–EDTA–sugar complexes were proposed as efficient specific enzyme-desorbing eluents.

It has been shown [32] that the influence of different metal ions differs depending on the nature of the dye and protein studied and that the immobilized and free dyes do not show the same mechanism towards the metal ion-mediated dye–enzyme association–dissociation [35] (ref. 37, p. 216). Further studies to elucidate the nature of transition metal ion-mediated binding of dyes to enzymes are necessary.

It was assumed [30] that the orientation of the dye–metal ion complex may be slightly different than that of the dye alone or the dye conformation could be stabilized owing to its coordination with metal ions in such a manner that the dye–metal ion complex becomes acceptable to the complementary protein [32]. This could lead to the formation of a highly selective ternary complex involving dye, metal ion and the enzyme at the active site region of the enzyme [33,34]. An analogous assumption has been made for the interaction of yeast alcohol dehydrogenase with the Cu(II) complex of Light Resistant Yellow 2KT [16,29]. Interaction of the free dye with the enzyme modified by diethyl pyrocarbonate, studied by means of differential and induced circular dichroism spectroscopy, confirmed the proposed assumption that the formation of a specific ternary complex involving dye, Cu^{2+}ions and histidine residue of yeast ADH analogous to His^{51} in horse liver ADH may be possible. The metal ion-promoted specific interaction of some dyes with yeast ADH has been confirmed by the results of enzyme partitioning in aqueous two-phase systems and this technique has enabled us to show [15] that the interaction of horse liver enzyme with some dyes depends also on the presence of Cu^{2+}ions. However, the results reported here show that the presence of Cu^{2+}ions in the structure of the dye could have an inverse action by lowering the specificity of the dye to the enzyme. This is supported by lactate dehydrogenase affinity partitioning in the presence of PEG conjugates with the Cu(II) complex of Red-Brown 2KT and its analogue with no metal ion when chelating agents and nucleotide ligand participated in two-phase systems (Tables III and IV, Figs. 1–3).

One can assume that the studied Cu(II) complexes of Light Resistant Yellow 2KT, Red-Violet 2KT in the case of LDH and Cu(II) complexes of Red-Brown 2KT, Claret 4CT and Red-Violet 2KT in the case of yeast G6PDH may interact predominantly at the coenzyme binding domains of the enzymes studied. This proposal is supported by the fact that the $\Delta\log K$ values of the enzymes are effectively decreased by low concentrations (up to 1 m*M*) of nucleotide ligands (Tables III and V). The same may be valid for the interaction of Red-Brown 2KT–Cu (II) after Cu^{2+}ions had been removed wih LDH (Fig. 3, Table III). If it is assumed that the same donor ligand from a nucleotide binding site was involved in the coordination with Cu^{2+}ions of dyes, it seems likely that the differences in the contribution involved in the binding with the enzyme coordination bonds are determined by the differences in the spatial structures of the dye–Cu(II) complexes. It is conceivable that the steric accessibility of Cu^{2+}ions coordinated with dyes determines the contribution of coordination bonds involved in the interaction with accessible donor groups of the enzyme.

It is well established that the accessibility of surface-exposed histidine residues in proteins governs the binding strength of immobilized metal chelates to proteins [40] and increases the partition coefficients of proteins in the presence of Cu(II) iminodiacetate (IDA)-derivatized poly(ethylene glycol) in PEG–dextran two-phase system [41,42]. As was mentioned above, such a coordination of histidine residue from yeast ADH with the Cu(II) complex of Light Resistant Yellow 2KT was supported by us. Unfortunately, at present we do not have exact information about the possibility of participation in coordination with metal ions of exposed histidine residue of LDH and G6PDH. With G6PDH, such participation of histidine residue is possible in view of the fact indicated by Kopperschläger and Lorez [26] that the imidazole residue of the enzyme could be involved in the interaction of Procion Navy HE-R-PEG with the enzyme. Earlier we concluded [16] that the differences in the interaction of LDH from rabbit muscle and yeast ADH with Light Resistant Yellow 2KT–Cu(II) may be due to the fact that in contrast to ADH a histidine residue with the same function is not observed at the nucleotide binding site of LDH. This conclusion agrees with the recent observation of Kirchberger *et al.* [24] that the hydrophobic nicotinamide pocket and arginine residue of the substrate binding site of LDH are probably involved in the binding with dyes such as Cibacron Blue F3G-A and Procion Red HE-3B.

As the coordination of the dye with metal ions could stabilize its conformation and limit conformation freedom, preference should be given to the differences in the conformational states of the studied dyes–Cu(II) complexes in their interaction with the enzyme. According to this, one could assume that the coordination of Red-Brown 2KT with Cu^{2+} may result in a dye conformation that would be expected to be spatially unfavourable for the spe-

cific interaction with LDH from rabbit muscle.

Further studies are necessary to support these assumptions but it is fairly clear that the direct confirmation of such interactions could be obtained by X-ray studies, as was determined [43] for the binding of Cibacron Blue F3GA to liver alcohol dehydrogenase.

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