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Interactions of human alkaline phosphatase isoenzymes with triazine dyes using affinity partitioning, affinity chromatography and difference spectroscopy

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ABSTRACT

Aqueous two-phase systems consisting of dextran, polyethylene glycol and dye-liganded polyethylene glycol were employed to investigate the affinity partitioning behaviour of isoenzymes of human alkaline phosphatase. Whereas in the system without a dye ligand the partition coefficients of the isoenzymes from human intestine and placenta were identical, the isoenzyme from human liver showed a significantly lower partition coefficient under the same conditions. After addition of dye-liganded polyethylene glycol two groups of dyes possessing substantial affinities to the isoenzymes were found. One, represented by Procion Yellow HE-3G, interacts specifically with the active centre of the isoenzymes. Differences in the affinity of the isoenzymes towards the individual dye ligands are caused only by the carbohydrate content, especially by the terminal sialic acid residues. The other group of dye ligands, represented by Procion Navy MX-RB, binds obviously in a more complex fashion involving other binding sites, which are only present in alkaline phosphatase of human liver. Procion Navy MX-RB was found to function as a suitable affinity ligand for the separation of human liver alkaline phosphatase from the other isoenzymes. Differences in the primary structure of two allelic forms of human placental alkaline phosphatase [(SS) and (F)] are not recognized in aqueous two-phase systems with or without dye-liganded polyethylene glycol.

INTRODUCTION

The abundance of alkaline phosphatases (AP), (EC 3.1.3.1) in living organisms, including bacteria, plants and animals, indicates that these enzymes are involved in fundamental biochemical processes, although the physiological function(s) are not clear [1]. At present, human AP isoenzymes can be classified into at least four groups with respect to biochemical and immunochemical properties. The isoenzymes are encoded by four distinct genes, positioned near the end of the long arm of chromosome 2 (q 34—37) for the intestinal (h-IAP), placental (h-PLAP) and the placental-like APs and near the end of the short arm of chromosome 1 (p 34–36.1) for the tissue-unspecific type (liver/bone/kidney/first-trimester placenta) isoenzyme [2].

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In routine clinical practice, AP is commonly used for the diagnosis and monitoring of bone and hepatobiliary diseases. In addition, as a tumour-associated marker, elevated serum AP activity can be helpful for differential diagnosis [3,4]. Discrimination of AP isoenzymes, still in the hands of few specialized clinical researchers, is based on differences in the thermal stability, inhibition and immunochemical properties and on differences in affinity to lectins [3,5,6]. However, there are still some points which need further elucidation, such as the true function of AP, the physiological significance of the oligosaccharide chains coupled to the enzyme and the transformation process of the membrane bound enzyme into a soluble form.

In this work, affinity partitioning in aqueous two-phase systems, affinity chromatography and difference spectroscopy were applied to study the binding behaviour of three human isoenzymes of AP to selected triazine dyes, which share the property to interact pseudo-biospecifically with the active site of calf intestinal AP (c-IAP) [7,8].

Dye-affinity partitioning has been found to be a sensitive approach to the study of ligand–protein interactions [9,10]. In order to characterize the effect of affinity partitioning, the difference in the logarithms of the partition coefficients of the enzymes in systems with and without a ligand, expressed as $\Delta \log K$ was calculated. When plotted against the concentration of dye-liganded polyethylene glycol (dye–PEG), this parameter usually follows a saturation function from which two parameters, *i.e.*, the maximum partitioning effect ($\Delta \log K_{max}$) and the relative affinity of the ligand to the enzyme ($0.5\Delta \log K_{max}$), have been estimated [11].

The comparative employment of affinity partitioning, difference spectroscopy and affinity chromatography reveals information on the chemical basis of the interaction between dye ligands and the protein and allows the study of the action of competing effectors, which indicates the specificity of binding [9,12,13].

EXPERIMENTAL

Materials

Procion dyes were obtained from ICI Organics Division (Blackley, UK) and Cibacron dyes from Ciba Geigy (Basle, Switzerland). Vilmax Dye I was a generous gift from Dr. Mazza (Vilmax, Buenos Aires, Argentina). The dyes were deactivated and purified according to Lowe and Pearson [14] before use in the difference spectroscopic experiments. Neuraminidase (*Clostridium perfingens*) was obtained from Sigma (Deisenhofen, Germany). Polyethylene glycol, ethylene glycol, DEAE-cellulose and 4-nitrophenyl phosphate were purchased from Serva (Heidelberg, Germany). Dextran T 70, Sepharose 4B and Superose 12 were products from Pharmacia (Uppsala, Sweden). All other biochemicals were of analytical-reagent grade.

Preparation of the immobilized dye derivatives

Dye–PEG. Triazine dyes were covalently coupled to PEG 6000 in aqueous alkaline solution and the dye–PEG derivatives were purifed by extraction with chloroform and ion-exchange chromatography on DEAE-cellulose according to Johansson [10]. The purity of the conjugates was determined by thin-layer chromatography on silica gel G 60 plates (Merck, Darmstadt, Germany) in 1-butanol–2-propanol–ethyl acetate–water (20:35:10:35, v/v).

Dye–Sepharose. Procion Yellow HE-3G and Procion Navy MX-RB were coupled to Sepharose 4B as described by Hughes *et al.* [15].

Enzyme preparations

Adult h-IAP and h-LAP were prepared from fresh human tissues (small intestine, liver), obtained from autopsies, by homogenization, extraction with *n*-butanol, acetone precipitation, DEAE ion-exchange chromatography and immunosorption. For h-PLAP(SS) and h-PLAP(F) the same scheme was used but additionally preparative isoelectric focusing and gel permeation were applied as described previously [16–19]. The specific activities of the purified isoenzymes were h-PLAP(SS) = 533, h-PLAP(F) = 395, h-IAP = 1400 and h-LAP = 4055 U mg^{-1}.

Enzyme assay

Alkaline phosphatase isoenzymes were assayed at 25°C with 4-nitrophenyl phosphate in 1 *M* diethanolamine–HCl buffer (pH 9.8) containing

1 m*M* $MgCl_2$ as described by Hausamen *et al.* [20]. One unit of activity is defined as the amount of enzyme that converts 1 μmol of substrate per minute at 25°C. The protein concentration of the purified enzymes was determined spectrophotometrically at 280 nm based on $A_1^{1\ \frac{mg}{cm}\ ml^{-1}} = 10.0$

Neuraminidase treatment

The enzyme digestion was carried out by incubation of 100 units of the respective isoenzyme and 100 milliunits of neuraminidase at 4°C in a total volume of 0.15 ml of 10 m*M* Tris–HCl buffer (pH 6.9), 2 m*M* $MgCl_2$ for 12 h. The sialic acid and the neuraminidase were removed by high-performance liquid chromatography on a Superose 12 (H10/30) column. Complete desialylation was checked by polyacrylamide gel electrophoresis under non-denaturing conditions [21].

Aqueous two-phase partitioning

Two-phase systems were prepared from stock solutions of PEG 6000 (20%, w/w), dextran T 70 (30%, w/w), Tris–HCl buffer (0.2 *M*), $MgCl_2$ (40 m*M*) (pH 7.5) and potassium phosphate (50 m*M*). The polymer concentrations are given as percentages of the total system. The amount of dye-liganded PEG given in percent is referred to the total mass of PEG present in the system. A 2-g sample of a two-phase system containing about 5 units of alkaline phosphatase was brought to 25°C and equilibrated by gently mixing for 30 s. After centrifugation at 1500 *g* for 5 min, samples for assay were withdrawn from both phases. Inhibition of the enzyme in the assay by the dye–PEG was avoided 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 phase.

Affinity chromatography

Disposable columns (40 × 8 mm I.D.) (Biorad Labs., Munich, Germany) containing 1.0 ml bed volume of dye-liganded Sepharose 4B were equilibrated with 10 m*M* Tris–HCl buffer (pH 7.5) containing 2 m*M* $MgCl_2$, at 25°C. The dialysed enzyme was applied in excess to the column and the unbound enzyme was washed out with equilibration buffer at a flow-rate of 30 ml h^{-1}. The bound enzyme was eluted with equilibration buffer containing different effectors.

Difference spectroscopy

Difference spectroscopy was performed with a Specord M40 double-beam spectrophotometer (Carl Zeiss, Jena, Germany) using 10 m*M* Tris–HCl buffer (pH 7.5) containing 2 m*M* $MgCl_2$ at 25°C. The light path of the cuvettes was 10 mm and the spectra were recorded at a scan rate of 2 nm s^{-1} and a constant slit width of 0.5 nm. The difference spectra were recorded after adding the same amount of dye to the reference and sample cells, respectively, with effector and enzyme concentrations as given under Results. The dye concentrations were determined spectrophotometrically by using the following molar absorptivities: Procion Yellow HE-3G (400 nm) 35 600 and Procion Navy MX-RB (581 nm) 16 700 l mol^{-1} cm^{-1}.

RESULTS

Affinity partitioning

In the absence of dye ligands, all isoenzymes partition in favour of the dextran-rich bottom phase ($\log K < -0.74$). The partition coefficients of h-IAP and h-PLAP were identical, but differ significantly from those of h-LAP (Table I). No significant difference in the affinity partitioning between h-PLAP(SS) and h-PLAP(F) was found. Therefore, the results obtained with these two allelic forms were designated as "h-PLAP". The partitioning of the isoenzymes in the presence of 2.5% of dye-liganded PEG is also shown in Table I. The affinity partitioning effect is minor with h-PLAP. Only Procion Navy H-ER, Procion Yellow HE-3G and Procion Green H-4G generate a measureable increase in the partition coefficient after addition of the dye ligand.

On the other hand, h-LAP and h-IAP show a substantial affinity partitioning with diverse dye ligands. When Procion Blue MX-G, Procion Blue MX-2G, Procion Red HE-3B, Procion Yellow HE-3G and especially Procion Navy MX-RB and Procion Blue H-5R were applied, differences in the extent of the partioning between the liver and the intestinal type were also found. Other dye ligands (Procion Red H-3B, Procion Scar-

TABLE I

AFFINITY PARTITIONING OF ISOENZYMES OF HUMAN ALKALINE PHOSPHATASE

The systems (2 g) containing 9.75% (w/w) dextran T 70, 6.5% (w/w) PEG 6000 (2.5% dye-liganded PEG), 10 m*M* Tris–HCl buffer (pH 7.5), 2 m*M* $MgCl_2$ and 5 units of AP activity were equilibrated at 25°C.

Dye–PEG	Log *K*		
	h-LAP	h-IAP	h-PLAP
Without	−1.22	−0.74	−0.76
Without (after desialylation)	−1.10	−0.75	−0.74
Procion Red HE-3B	−1.22	−0.38	−0.75
Procion Red MX-8B	−1.12	−0.48	−0.75
Procion Red H-3B	−1.22	−0.72	−0.75
Procion Scarlet MX-G	−1.22	−0.67	−0.73
Procion Orange MX-2R	−1.17	−0.74	−0.75
Procion Blue MX-2G	−1.14	−0.37	−0.72
Procion Blue MX-G	−0.83	−0.10	−0.75
Procion Blue H-5R	−0.04	−0.37	−0.75
Procion Blue MX-3G	−0.88	−0.48	−0.75
Procion Navy H-ER	−0.57	−0.34	−0.51
Procion Navy MX-RB	−0.20	−0.57	−0.75
Procion Yellow HE-3G	−0.61	+0.35	−0.44
Procion Yellow H-3R	−1.08	−0.55	−0.72
Procion Brown H-4GR	−1.19	−0.73	−0.75
Procion Green H-4G	−0.80	−0.05	−0.55
Vilmax Dye I	−1.14	−0.55	−0.72
Cibracron Red 3BA	−1.10	−0.67	−0.65
Cibacron Blue F3G-A	−1.16	−0.54	−0.64

let MX-G, Procion Orange MX-2R and Procion Brown H-4GR) do not alter the partitioning of the human AP-isoenzymes.

In order to study the influence of the sialic acid residues of the isoenzymes on the dye–enzyme interaction, neuraminidase-treated and untreated forms of the respective isoenzymes were partioned. In the systems without dye-liganded PEG the partition coefficients of the isoenzymes were not changed by treatment with neuraminidase (Table I).

As shown in Fig. 1, neuraminidase treatment of h-IAP does not change the affinity partitioning ($\Delta\log K$) in the presence of six selected dyes. However, the $\Delta\log K$ for h-PLAP and h-LAP increased after desialylation and at 2.5% dye–PEG achieved values similar to or higher than those for h-IAP as demonstrated for Procion Blue H-5R and Procion Navy MX-RB.

Fig. 2 shows the affinity partitioning of the neuraminidase-treated and untreated h-LAP as a function of the concentration of Procion Navy MX-RB- and Procion Yellow HE-3G-liganded PEG as an example. The calculation of the maximum affinity partitioning ($\Delta\log K_{max}$) revealed that this parameter is not influenced by the treatment of any isoenzymes with neuraminidase (Table II). On the other hand, the relative affinity ($0.5\Delta\log K_{max}$) of h-LAP and h-PLAP decreased drastically and achieved the value for h-IAP in the case of Procion Yellow HE-3G (Table II).

Because of the missing saturation function of the partition curves of h-IAP and h-PLAP in the presence of Procion Navy MX-RB-PEG under the conditions used, the $\Delta\log K_{max}$ and the relative affinity were not calculable.

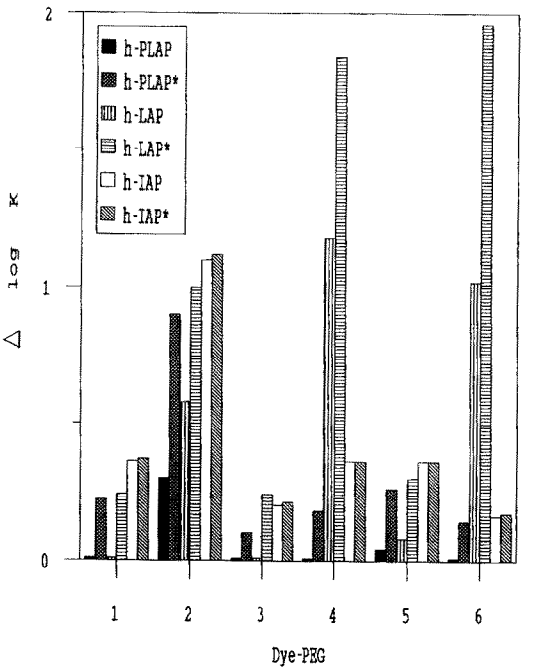

Fig. 1. Dye ligand affinity partitioning of isoenzymes of human alkaline phosphatase before and after treatment with neuraminidase. The systems (2 g) containing 9.75% (w/w) dextran T 70, 6.5% (w/w) PEG 6000 (2.5% dye-liganded PEG), 10 m*M* Tris HCl buffer (pH 7.5), 2 m*M* $MgCl_2$ and 5 units of the respective isoenzyme were equilibrated at 25°C. (1) Procion Red HE-3B; (2) Procion Yellow HE-3G; (3) Cibacron Blue F3G-A; (4) Procion Blue H-5R; (5) Procion Blue MX-2G; (6) Procion Navy MX-RB. ⋆ = Neuraminidase-treated form of the isoenzyme. The log *K* values of the isoenzymes in the absence of dye-liganded PEG are given in Table I.

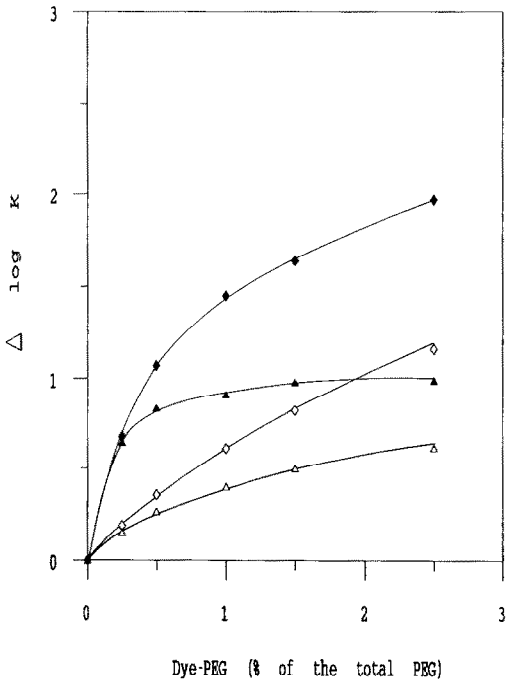

Fig. 2. Dye ligand affinity partitioning of alkaline phosphatase of human liver as a function of the concentration of the dye–PEG. The systems (2 g) containing 9.75% (w/w) dextran T 70, 6.5% (w/w) PEG 6000 (partially replaced with dye-liganded PEG), 10 m*M* Tris–HCl buffer (pH 7.5), 2 m*M* $MgCl_2$ and 5 units of the isoenzyme were equilibrated at 25°C. Open symbols, h-LAP; closed symbols, h-LAP after desialylation. Procion dyes: △, ▲ = Yellow HE-3G; ◇,◆ = Navy MX-RB. The log *K* values of the isoenzymes in the absence of dye-liganded PEG are given in Table I.

In order to study the dye–isoenzyme interaction with respect to the specificity of binding, the effect of phosphate on the extent of affinity partitioning of the isoenzymes was analysed. As can be seen in Table III, the effect of phosphate depends on the kind of dye and the kind of isoenzyme. The competition of phosphate on the partitioning of neuraminidase-treated and untreated h-IAP in the presence of Procion Yellow HE-3G-PEG was the same.

At 5 m*M* phosphate the affinity partitioning of h-PLAP and h-LAP in the presence of Procion Yellow HE-3G was completely abolished. However, the treatment of these isoenzymes with neuraminidase reduced the competition of phosphate. In the system containing Procion Navy MX-RB, 0.5 m*M* phosphate is capable of abolishing the affinity partitioning of h-IAP completely. However, the effect is less pronounced with h-LAP, especially with the desialylated form of this isoenzyme.

TABLE II

AFFINITY PARTITIONING OF ISOENZYMES OF HUMAN ALKALINE PHOSPHATASE AS A FUNCTION OF THE CONCENTRATION OF DYE-LIGANDED PEG IN THE SYSTEM

The systems (2 g) containing 9.75% (w/w) dextran T 70, 6.5% (w/w) PEG 6000 (2.5% dye-liganded PEG), 10 m*M* Tris–HCl buffer (pH 7.5), 2 m*M* $MgCl_2$ and 5 units of AP activity were equilibrated at 25°C.

Enzyme	Procion Yellow HE-3G		Procion Navy MX-RB	
	$\Delta\log K_{max}$	Relative affinity[a]	$\Delta\log K_{max}$	Relative affinity[a]
h-IAP	1.08	0.15	Values are not calculable under the conditions used.	
h-IAP[b]	1.10	0.15		
h-PLAP	0.94	1.32	As above	
h-PLAP[b]	0.98	0.20		
h-LAP	0.95	1.21	2.70	3.46
h-LAP[b]	1.05	0.15	2.65	0.77

[a] Concentration of dye–PEG (% of the total PEG) yielding 50% of the $\Delta\log K_{max}$.
[b] Neuraminidase-treated form of the isoenzyme.

The effect of pH on the partitioning of h-LAP in aqueous two-phase systems is demonstrated in Fig. 3. Whereas in systems without a dye ligand the partioning was hardly influenced between pH 7.5 and 9.5, the partition coefficients of h-LAP decreased with increasing pH in the presence of Procion Yellow HE-3G and Procion Navy MX-RB. Especially with Procion Yellow HE-3G a strong change in the partitioning of h-LAP between pH 7.5 and 8.0 was observed.

Affinity chromatography

For the affinity chromatography of h-LAP, Procion Navy MX-RB and Procion Yellow HE-3G were selected and covalently coupled to Sepharose 4B. The abilities of diverse effectors to desorb the bound enzyme are summarized in Table IV. h-LAP is eluted from Procion Yellow HE-3G–Sepharose with more than 90% recovery

TABLE III

AFFINITY PARTITIONING OF ISOENZYMES OF HUMAN ALKALINE PHOSPHATASE AS A FUNCTION OF THE PHOSPHATE CONCENTRATION (0.5 AND 5.0 m*M*) IN THE SYSTEM

The system (2 g) containing 9.75% (w/w) dextran T 70, 6.5% (w/w) PEG 6000 (2.5% dye-liganded PEG), 10 m*M* Tris–HCl buffer (pH 7.5), 2 m*M* $MgCl_2$ and 5 units of AP activity were equilibrated at 25°C.

Enzyme	Residual $\Delta \log K$ (%)[a]			
	Procion Yellow HE-3G		Procion Navy MX-RB	
	0.5 m*M*	5.0 m*M*	0.5 m*M*	5.0 m*M*
h-IAP	93	34	0	0
h-IAP[b]	92	31	0	0
h-PLAP	40	0	n.d.[c]	n.d.
h-PLAP[b]	90	25	n.d.	n.d.
h-LAP	42	0	84	56
h-LAP[b]	85	22	100	86

[a] The $\Delta \log K$ obtained in the absence of phosphate was taken as 100%.

[b] Neuraminidase-treated form of the isoenzyme.

[c] No competition detectable because $\Delta \log K < 0.05$.

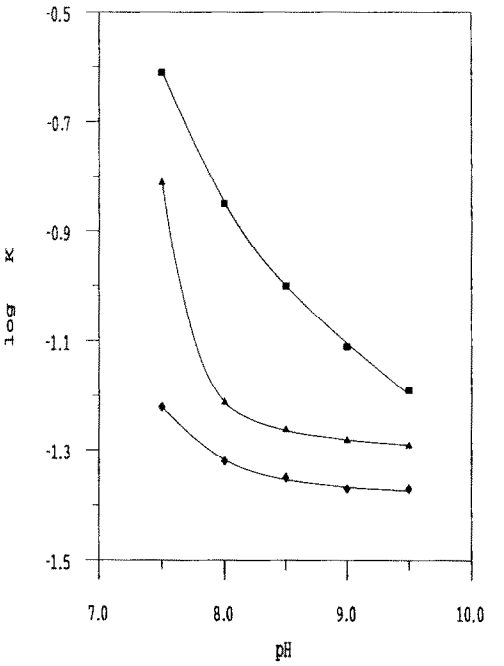

Fig. 3. Affinity partitioning of h-LAP as a function of pH. The systems (2 g) containing 9.75% (w/w) dextran T 70, 6.5% (w/w) PEG 6000 (1% dye-liganded PEG), 10 m*M* Tris–HCl buffer (pH 7.5–9.5), 2 m*M* $MgCl_2$ and 5 units of the isoenzyme were equilibrated at 25°C. Procion dyes: ▲ = Yellow HE-3G; ■ = Navy MX-RB; ◆ = without dye–PEG.

by applying 5 m*M* phosphate, by increasing the ionic strength and the pH of the buffer and by using a hydrophobic effector (ethylene glycol). In contrast, no desorption of h-LAP from Procion Navy MX-RB–Sepharose was found by using these effectors or EDTA. Only by increasing the pH the elution of h-LAP was achieved, independently of the buffer used.

The two dye ligands immobilized on Sepharose 4B were also able to bind h-IAP and h-PLAP, but with different binding capacities (data not shown). The complete elution of both isoenzymes was achieved with 0.5 *M* NaCl (data not shown).

Difference spectroscopy

The absorption spectrum of Procion Yellow HE-3G in Tris–HCl buffer (pH 7.5) displayed a maximum at 405 nm. As shown in Fig. 4A, a hypochromic deviation with two minima at 366 and 410 nm was recorded in the presence of NaCl. In the presence of ethylene glycol a hyperchromic deviation with a positive peak at 420 nm was obtained. The difference spectrum in the presence of NaCl can be regarded as an "ionic" and in the presence of ethylene glycol as a "hydrophobic" spectrum [12].

The absorption spectrum of Procion Navy MX-RB in Tris–HCl buffer (pH 7.5) showed a maximum at 581 nm. The "ionic" spectrum of this dye exhibited hypochromicity with a bathochromic shift of the absorption maximum to 659 nm and a minimum at 585 nm. The "hydrophobic" spectrum of this dye also showed a bathochromic shift with a maximum at 633 nm and a minimum at 544 nm (Fig. 4B). The shape of the difference spectrum of Procion Yellow HE-3G in the presence of h-LAP is very similar to the "hydrophobic" spectrum of this dye (Fig. 4A). An unambiguous classification of the difference spectrum of Procion Navy MX-RB in the presence of h-LAP is not possible; hydrophobic and electrostatic interactions are reflected (Fig. 4B).

TABLE IV

AFFINITY CHROMATOGRAPHY OF HUMAN LIVER ALKALINE PHOSPHATASE (h-LAP)

The dye-liganded Sepharose was equilibrated with 10 m*M* Tris–HCl buffer (pH 7.5), 2 m*M* $MgCl_2$ at 25°C. Ten units of h-LAP were applied and the unbound activity was removed by washing with equilibration buffer.

Effector[a]	Enzyme activity eluted[b] (%)		Conductivity (mS cm^{-1})
	Procion Navy MX-RB	Procion Yellow HE-3G	
Ethylene glycol (50%, v/v)	0	98	0.95
NaCl (0.25–1.5 *M*)	0	98	18–120
Potassium phosphate (5 m*M*)	0	91	1.99
EDTA (10 m*M*)	0	n.d.[c]	2.75
KSCN	0	95	21
Diethanolamine–HCl (10 m*M*):			
pH 7.5	33	38	0.91
pH 9.5	98	98	0.16
2-Amino-2-methyl-1,3-propanediol–HCl (pH 9.5) (10 m*M*)	97	98	0.13

[a] The effectors were dissolved in the equilibration buffer and the pH was adjusted to 7.5.
[b] The bound enzyme activity (calculated from the difference of the loaded and washed out activity) was taken as 100%.
[c] Not detected.

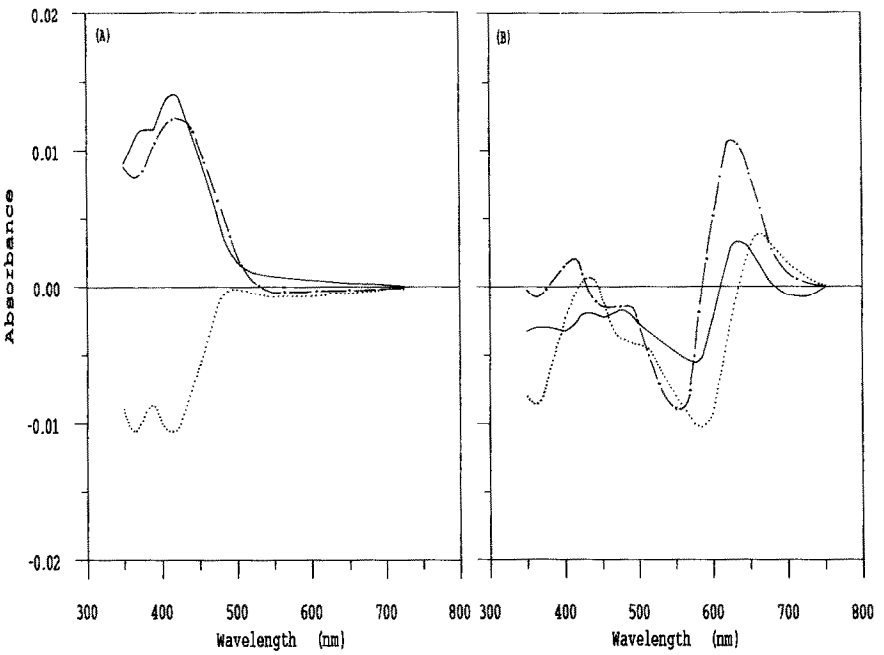

Fig. 4. Difference spectroscopy of (A) Procion Yellow HE-3G and (B) Procion Navy MX-RB dissolved in 10 m*M* Tris–HCl buffer (pH 7.5), 2 m*M* $MgCl_2$ at 25°C. The sample cuvette contained (dotted line) 0.5 *M* NaCl at 7.0 μM Procion Yellow HE-3G and 50.5 μM Procion Navy MX-RB; (dot-dashed line) 50% (v/v) ethylene glycol at 7.0 μM Procion Yellow HE-3G and 10.5 μM Procion Navy MX-RB; and (solid line) 0.133 mg ml^{-1} h-LAP at 20.0 μM Procion Yellow HE-3G and 17.0 μM Procion Navy MX-RB.

DISCUSSION

The method of partitioning in aqueous two-phase systems consisting of dextran and PEG is able to distinguish between h-AP isoenzymes. Differences in the primary structure of h-LAP and the two other isoenzymes (only 57% homology in the amino acid sequence with h-PLAP and 86% homology between h-IAP and h-PLAP [22] could influence the degree of surface hydrophobicity and therefore determine the partitioning in aqueous two-phase systems. This is also indicated by the stronger binding of this isoenzyme to hydrophobic matrices [23].

On the other hand, h-IAP and h-LAP partition in a similar manner, although different epitopes for h-IAP and h-PLAP are detectable [24] and separation of both isoenzymes by applying hydrophobic interaction chromatography does occur [23]. Similarly, the partitioning of the two allelic forms of the placenta type [h-PLAP(SS) and h-PLAP(F)] which contain only seven amino acid exchanges [25], was the same.

The applicability of dye–enzyme interactions for the discrimination of isoenzymes requires the selection of a suitable ligand, as already reported for the isoenzymes of lactate dehydrogenase [26].

The interaction of multiple forms of mammalian alkaline phosphatases with diverse triazine dyes has been studied preferentially by using kinetic and chromatographic methods [7,8,27–31]. As shown under Results, only a distinct group of dye ligands is able to distinguish between the h-AP isoenzymes.

Dye ligands which generate a relative high affinity partitioning effect with h-IAP showed similar results to the enzyme from calf intestine [32].

The lowest affinity to all the dye ligands screened was found for h-PLAP. As shown by different methods, most of the dye ligands bind specifically close to the phosphate-binding site of AP from different sources [13,27,28]. However, the primary structure of the putative active centre of h-IAP and h-PLAP is nearly identical regarding the substrate/phosphate binding site (only one substitution: $Ala_{93} \leftrightarrow Gly_{93}$) and the distribution of arginine which is involved in the phosphate binding of h-PLAP [22,33].

As h-PLAP, in contrast to h-IAP, contains oligosaccharides with terminal sialic acid residues [21] and the desialylation of h-PLAP caused an increase in the affinity to the enzyme without changing $\Delta \log K_{max}$, the difference in the affinity partitioning of h-PLAP and h-IAP is obviously caused by the carbohydrate moiety of h-PLAP. This assertion agrees with results of Mössner *et al.* [34], who found that sialic acid residues produce an electrostatic barrier for the binding of L-histidyldiazobenzylphosphonic acid.

The results in this paper reveal that the binding of Procion Yellow HE-3G is similar for all isoenzymes and directed to the active centre of AP, as already reported for Cibacron Blue F3G-A and Remazol Yellow GGL [27,28]. The partitioning behaviour of h-LAP in the presence of Procion Navy MX-RB-PEG and the weak competitive effect of phosphate point to another binding mechanism, however. With Procion Yellow HE-3G, difference spectroscopic experiments showed dominating hydrophobic interactions between the dye and h-LAP. On the other hand, the results of affinity chromatography and the influence of pH on the partitioning indicated that weak electrostatic forces cannot be ruled out.

The interaction between Procion Navy MX-RB and h-LAP is probably more complex. Difference spectroscopy showed that both hydrophobic and electrostatic binding forces are involved. Consequently, neither 0.25–1.5 *M* NaCl, hydrophobic agents nor chaotropic ions are able to dissociate the dye–enzyme complex. An increase in pH weakens the electrostatic interaction between the dye and h-LAP without changing the ionic strength. An active site-directed influence of diethanolamine, which is able to act as a transphosphorylating buffer [35], can be excluded.

As Procion Navy MX-RB contains copper, the formation of dye–metal–enzyme complex could be possible [36]; however, EDTA did not dissociate the complex.

The interaction of Procion Navy MX-RB with h-LAP points to a high degree of unspecific bindings, which only exist in this type of h-AP isoenzyme.

As Procion Navy MX-RB–Sepharose exhibits an ability to bind even at high ionic strength only h-LAP and the bound isoenzyme can be eluted with high recovery by increasing the pH, this dye

ligand seems to be a favourable canditate for the determination of the proportion of h-LAP in the total activity in human body fluids, which is of diagnostic relevance. However, the influence of individual proteins in blood plasma on the binding capacity of the dye-liganded Sepharose should be determined in additional experiments.

In conclusion the method of partitioning in aqueous two-phase systems is able to recognize differences in the physico-chemical properties of h-AP isoenzymes. Among different dye ligands, two groups can be distinguished. One, represented by Procion Yellow HE-3G, is able to interact specifically with the active centre of h-AP isoenzymes. The other, represented by Procion Navy MX-RB, binds less specifically at different binding regions, which are only present in h-LAP. The structural delineation of the two groups of dye-ligands has not been possible so far. The interaction between the isoenzymes and dye ligands of both groups is influenced by terminal sialic acid residues. The differences in the primary structures of h-PLAP(SS) and h-PLAP(F) are not recognized in dye ligand affinity partitioning.

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