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AFFINITY PARTITIONING OF ENZYMES USING DEXTRAN-BOUND PROCION YELLOW HE-3G

INFLUENCE OF DYE–LIGAND DENSITY

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SUMMARY

The dye Procion Yellow HE-3G was bound to dextran of molecular weight 70 000 and the partitioning of this dye–polymer within an aqueous two-phase system containing dextran and poly(ethylene glycol) was studied as function of ligand density, polymer concentration, type of salt, concentration of salt and concentration of dye–dextran. Even moderate dye:dextran molar ratios (5–8) make the partitioning strongly salt-dependent. The dye–dextran can be directed to either the upper or the lower phase with partition coefficients from 0.02 to 28 by using salts. The dye–dextran in the two-phase system affects the partitioning of dye-binding enzymes (lactate dehydrogenase, glucose-6-phosphate dehydrogenase, 3-phosphoglycerate kinase) towards the dye-containing phase. Measurements of competition with nucleotide binding show an increased affinity of the dye for the enzyme with increasing ligand:dextran ratio. Theoretical considerations indicate that 1–2 dextran molecules are attached per enzyme molecule when affinity partitioning is fully developed.

INTRODUCTION

Aqueous (liquid–liquid) two-phase systems are formed when two polymers are dissolved together in water at high enough concentrations[1,2]. These two-phase systems have been used for the separation of a number of cell components and also cells and viruses by taking advantage of the different partitioning between the two phases (and/or the interface between them). The most commonly used pair of polymers has been dextran and poly(ethylene glycol) (PEG). Whereas PEG is concentrated in the lighter upper phase, dextran is found mainly in the lower phase[1]. Polymers have therefore been used as carriers for affinity ligands to localize them mainly in one phase. The introduction of affinity ligands has a profound and selective influence on the partitioning of the biological material included in the system. This principle (affinity partitioning[3]) has mostly been carried out with PEG-bound affinity ligands[3–10], but dextran has also been used in a few instances as a ligand carrier[11,12]. Reactive textile dyes have been used as affinity ligands for affinity partitioning of enzymes[11–17].

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The influence of dextran-bound Procion Yellow HE-3G on the partitioning of some enzymes has been studied in this work. The dependence of affinity partitioning on the number of binding sites per enzyme molecule and the degree of substitution of the ligand dextran have been investigated. The study also included the determination of the strength of the binding of dye–dextran to the enzymes relative to the normal nucleotides.

EXPERIMENTAL

Chemicals

Dextrans T-40, T-70, T-500 and T-2000 were purchased from Pharmacia (Uppsala, Sweden). Poly(ethylene glycol) (PEG) with M_r = 7000–9000 was obtained from BP Chemicals (Hythe) as Breox 8000. Dye–PEG was synthesized as described elsewhere[18], yielding a monosubstituted polymer. Procion Yellow HE-3G was a kind gift from Swedish ICI (Göteborg, Sweden). Lactate dehydrogenase from rabbit muscle, glucose-6-phosphate dehydrogenase and 3-phosphoglycerate kinase from yeast together with biochemicals for the enzyme assays were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical-reagent grade. The distilled water used was first passed through a mixed ion exchanger.

Synthesis of dye–dextran derivatives

Aqueous solutions (180 ml) of 9 g of dextran T-70 and Procion Yellow HE-3G (1.4, 2.2, 4.2 and 7.0 g) were heated to 70°C, then 18 ml of a solution containing 1 *M* sodium hydroxide and 0.5 *M* sodium sulphate were added to each reaction mixture. After 45 min at the same temperature the mixtures were neutralized by addition of concentrated acetic acid (2 ml to each). The solutions were allowed to cool to 30–35°C and the dextran was precipitated by slow addition of ethanol (300 ml) with stirring. The precipitates were dissolved in 200 ml of water and again precipitated with ethanol (300 ml). To remove unreacted dye the dextran was dissolved in 100 ml of water and then mixed with 30 ml of DEAE-cellulose ion exchanger (Whatman DE-52, suspension in water) and 5 ml of 2 *M* potassium chloride. After mixing for 3 h, the ion exchanger was removed by filtration and washed with 0.1 *M* potassium chloride. The filtrate (together with the wash liquid) was again treated with ion exchanger as above, keeping the salt concentration at 0.1 *M*, either once (for the two lower dye concentrations) or twice (for the two higher dye concentrations). The final filtrates were dialysed against hot tap water (4 h) and then against distilled water overnight. The solutions were concentrated by evaporation under vacuum to a final concentration of 5–10%.

The concentration of dextran was determined by polarimetry using the specific rotation[1] $[\alpha]_D^{20} = +199°$ ml g^{-1} dm^{-1}. The concentration of bound dye was determined by absorbance measurements at 400 nm assuming a molar absorptivity of 35 600 l mol^{-1} cm^{-1}. This value was derived from absorbance measurements of monosubstituted Procion Yellow HE-3G PEG. The substituted dextran (Procion Yellow HE-3G–dextran or PrY–dextran) contained 19, 33, 76 and 120 μmol g^{-1} of dye, respectively, which corresponds to a degree of substitution (n) of 1.3, 2.3, 5.3 and 8.3 dye molecules, respectively, per dextran molecule with an assumed molecular weight of 70 000.

Enzyme assays

The enzyme activities were determined photometrically at 340 nm and 22°C using a Hitachi 100-60 spectrophotometer. Lactate dehydrogenase (E.C. 1.1.1.27) was determined according to Bergmeyer[19], glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) according to Noltmann *et al.*[20] and 3-phosphoglycerate kinase (E.C. 2.7.2.3) according to Scopes[21].

Two-phase systems

The systems were prepared by weighing out the required amounts of solutions of 40% (w/w) PEG and 20 or 30% (w/w) dextran, together with salt, buffer and enzyme solution, plus water to the final weight (normally 4 g). The systems were equilibrated at 22°C, centrifuged for 1 min at 2000 *g* and samples of known volume were withdrawn and analysed. The partition coefficient of the enzyme, K, defined as the ratio between the enzyme activities in the upper and lower phases, was calculated. The partition coefficients of the free dye and of dye–dextran were determined by absorbance measurements at 400 nm using equally diluted phases without dye as blanks.

Enzyme precipitation

Mixtures containing lactate dehydrogenase (25 U ml^{-1}), PEG (80 g l^{-1}), sodium phosphate buffer (pH 7.9) (50 mM) and various concentrations of PrY–dextran (0–8 μM dye) were centrifuged 2 min at 7000 *g* at 22°C.

Competitive binding

Activity measurements on constant amounts of enzyme were carried out in the presence of various concentrations of dye–dextran, dye–PEG or free (deactivated) dye. The concentration of dye that gave 50% inhibition was determined. For 3-phosphoglycerate kinase, an increase in concentration of the auxiliary enzyme (glyceraldehydephosphate dehydrogenase) did not change the inhibition curve. The association constant of the dye to the enzyme is given relative to the respective nucleotide, *i.e.*, how many times more strongly the dye binds compared with the normal coenzyme.

RESULTS AND DISCUSSION

Partition of PrY–dextran

The free dye partitioned mainly to the upper phase (partition coefficient $K > 1$), but its partition was clearly influenced by the salt composition (Table I). PrY–dextran, on the other hand, distributed between the phases (in systems containing 50 mM sodium phosphate buffer, pH 7.9) with partition coefficients $K = 0.15$–24 depending on the molecular weight of dextran and the degree of substitution of PrY–dextran (Table II). The partition coefficient for PrY–dextran increased with increasing degree of substitution.

In the (liquid–liquid) two-phase systems formed by dextran and PEG in water solution the dextran is mainly found in the lower phase[1]. The partitioning of PrY–dextran may not necessarily be the same as that of the unsubstituted dextran because the bound dye introduces both hydrophobic and ionic elements into the macromol-

TABLE I

PARTITION OF PROCION YELLOW HE-3G

Partition coefficients, K, of free Procion Yellow HE-3G in various two-phase systems. K is defined as the ratio between the dye concentrations in the upper and lower phase. Temperature, 22°C. System composition: 8% (w/w) dextran 70, 4.5% (w/w) PEG 8000, 50 μM dye and salt. Measured pH in salt-containing system, 7.7–7.9; in salt-free system, 7.5.

Salt content	*K*
None	2.8
100 mM sodium perchlorate	1.4
100 mM potassium chloride, 5 mM sodium phosphate buffer (pH 7.9)	2.5
100 mM sodium acetate (pH 7.8)	2.9
100 mM sodium sulphate	6.4
50 mM sodium phosphate buffer (pH 7.9)	7.5

ecule. The affinity of free Procion Yellow HE-3G for the upper phase may be explained by the presence of aromatic elements in the dye which are known to be better solvated in PEG solutions than in pure water[22]. It is well known that the partitioning of macromolecules within PEG–dextran systems[1,2] is influenced by several factors, including the concentration and molecular weight of dextran and PEG, salt composition and pH.

The influence of the concentration of phase-forming polymers on the partitioning of PrY–dextran and free dye is shown in Fig. 1. The effect is in line with observations made on the partitioning of proteins[1,2,23]. It is known from earlier experiments[24] that the salt-steering effect increases with increasing polymer concentration. An increase in the molecular weight of dextran (in systems of comparable distance from the critical point) is another well known way of increasing the partition coefficients of proteins. The same effect was found here for the PrY–dextrans (Table II). The concentration of PrY–dextran had only a slight influence on its partition coefficient in the range 0.5–4 g l^{-1} of PrY–dextran (Table III).

Different salts showed strong and diverse effects on the partitioning of dye–dextran (Fig. 2). Whereas phosphate and sulphate increased the partition coefficient of PrY–dextran (with increasing degree of substitution), perchlorate had the opposite

TABLE II

EFFECT OF MOLECULAR WEIGHT OF DEXTRAN

Partitioning of PrY–dextran 70 in systems composed of PEG 8000, dextran of various molecular weights and 50 mM sodium phosphate buffer (pH 7.9). Temperature, 22°C. n = Degree of substitution.

Molecular weight of dextran	*Composition of system*		*K of PrY–dextran*			
	Dextran (%, w/w)	*PEG (%, w/w)*	*n = 1.3*	*n = 2.3*	*n = 5.3*	*n = 8.3*
40 000	8.00	5.00	0.15	0.30	2.0	17.5
70 000	8.00	4.50	0.23	0.50	4.1	28.2
500 000	5.00	3.80	0.97	1.62	6.2	24.3
2000 000	5.00	4.00	1.03	1.83	7.5	22.5

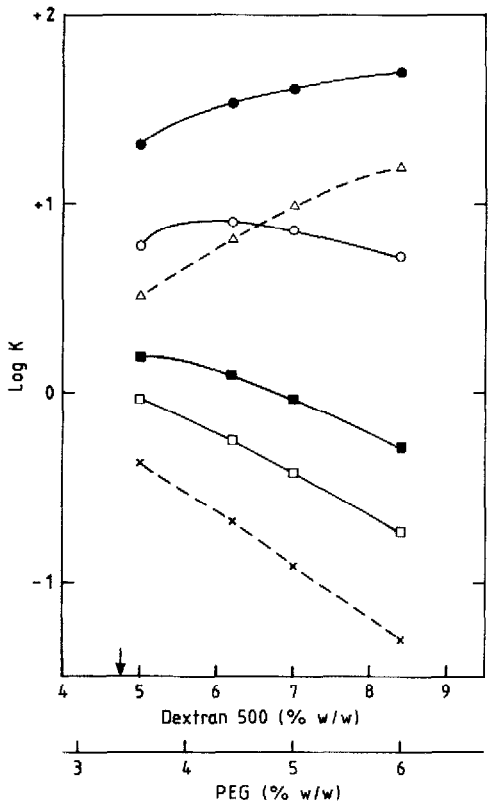

Fig. 1. Partition of Procion Yellow HE-3G and PrY–dextran 70 (50 μM dye) in phase systems containing PEG 8000 and dextran 500 as well as 50 mM sodium phosphate buffer (pH 7.9). Temperature, 22°C. △, Free dye; □, PrY–dextran (n = 1.3); ■, PrY–dextran (n = 2.3); ○, PrY–dextran (n = 5.3); ●, PrY–dextran (n = 8.3); and ×, unsubstituted dextran 70 (extrapolated values). Arrow indicates transition point between one and two phases.

TABLE III

CONCENTRATION DEPENDENCE OF THE PARTITION COEFFICIENT OF PrY–DEXTRAN 70

System: 8% (w/w) dextran 70, 4.5% (w/w) PEG 8000, 0.05–0.84% PrY–dextran 70 (n = 2.3) and 50 mM sodium phosphate buffer (pH 7.9). Temperature, 22°C.

Concentration of PrY–dextran		*K*	*Log K*
% (w/w)	*μM dye*		
0.05	18	0.51	−0.29
0.11	36	0.51	−0.29
0.21	72	0.49	−0.31
0.42	144	0.48	−0.32
0.84	288	0.45	−0.35

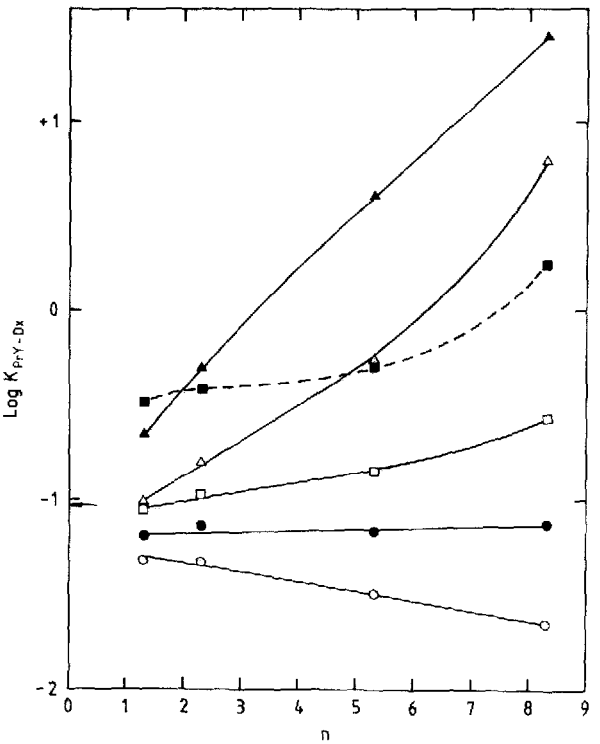

Fig. 2. Influence of salt on the partitioning of PrY–dextran 70. System composition: 8% (w/w) dextran 70, 4.5% (w/w) PEG 8000, 50 μM dextran-bound dye and salt. The pH values were adjusted to 7.9 with sodium hydroxide. ■, Salt-free system; ▲, 50 mM sodium phosphate buffer (pH 7.9); △, 100 mM sodium sulphate; □, 100 mM sodium acetate; ●, 100 mM potassium chloride + 5 mM sodium phosphate buffer (pH 7.9); and ○, 100 mM sodium perchlorate. Arrow indicates partition coefficient of unsubstituted dextran. Temperature, 22°C.

TABLE IV

PARTITION OF PrY–DEXTRAN AT VARIOUS CONCENTRATIONS OF SODIUM PHOSPHATE BUFFER

System: 8% (w/w) dextran 70, 4.5% (w/w) PEG 8000, 30 μM dextran-bound Procion Yellow HE-3G (PrY–dextran 70, n = 5.3) and sodium phosphate buffer (pH 7.5). Temperature, 22°C.

Concentration of phosphate buffer (mM)	*Partition coefficient of PrY–dextran*	
	K	*Log K*
2.5	3.34	0.52
5	3.40	0.53
7.5	7.81	0.89
10	6.48	0.81
25	4.85	0.69
50	3.41	0.53

effect. With potassium chloride the partitioning was almost independent of the degree of substitution. When PrY–dextran was partitioned in systems containing various concentrations of sodium phosphate buffer (Table IV), a remarkable dependence was observed. The partition coefficient of dextran-bound dye doubled when the phosphate concentration was increased from 5 to 7.5 m*M*. This may reflect a conformational change in the dye–dextran molecule, where intramolecular forces between the dye groups can play an important role.

Addition of salts (in excess) to an aqueous two-phase system is known to influence the partitioning of charged molecules between the phases. This phenomenon has been connected with the interfacial potential caused by the ions of the salt[1,2,23]. The dye should, according to the observed shift in log K, have a negative net charge of 7–10 units[23,25]. When bound to dextran in small amounts (degree of substitution $n = 1.3$), the dye is mainly restricted to the lower phase but the interfacial potential again influences the partitioning. When one ligand molecule is assumed to be bound per dextran, the change in log K is only half of that of the free dye. The reason could be a stronger tendency towards ion pair formation between sulphonic or carboxylic groups (present in the dye molecule) and the cations, *e.g.*, Na^+, owing to close contact between the dye and dextran segments. When these segments are part of the solvation shell of the dye, it is also possible that carboxylic and amino groups change their dissociation constants, which may give rise to a lower net charge. When several dye groups are bound to dextran, their cumulative net charge makes the partitioning of ligand–dextran increasingly dependent on the steering salt. Hence oligo-substituted dextran can be forced into the upper phase when certain salts (*e.g.*, sulphates and phosphates) are used.

Partitioning of enzymes

To evaluate the influence of PrY–dextran on the partitioning of dye-binding enzymes, three enzymes were used: lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G6PDH) and 3-phosphoglycerate kinase (PGK), containing four, two and one binding sites, respectively, for the nucleotide (NAD, NADP or ATP)[26–28]. The dye is assumed to bind to the same binding sites[29].

Fig. 3 shows the partitioning of LDH in systems containing various concentrations of PrY–dextran. At high concentrations of dye–ligand the partitioning of LDH approaches a constant (saturation) value. This is similar to what has been observed when dye–PEG is used[30]. At low concentrations of dye, however, the extraction curves do not show (except when $n = 1.3$) the monotonic appearance observed with PEG-bound ligands. A possible explanation could be a precipitating effect of the dye–dextrans. Such affinity precipitations have been reported for enzymes with several binding sites mixed with equimolar amounts of bis-ligand (NAD) derivatives[31]. To test this hypothesis, LDH was uncubated with PrY–dextran in the presence of PEG in concentrations such that no phase separation occurred (Fig. 4). Indeed, the precipitating effects increased with increasing degree of substitution and showed a maximum when the ratio between dextran-bound and nucleotide binding sites on LDH was 1.5–2.5. The fraction of enzyme in aggregated form may partition with a lower partition coefficient than the non-aggregated enzyme.

The influence of PrY–dextrans on the partitioning of G6PDH (Fig. 5) was similar to that found for LDH, but the precipitation was less pronounced and rela-

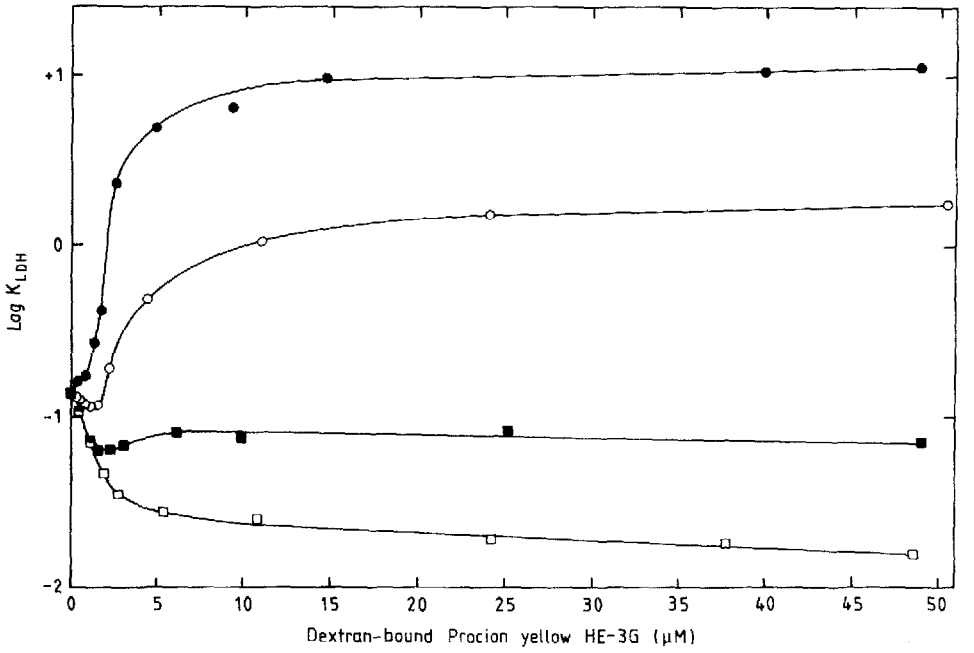

Fig. 3. Partitioning of lactate dehydrogenase (14 kU l^{-1}) in systems containing increasing concentrations of PrY–dextran. System: 8% (w/w) dextran 70, 4.5% (w/w) PEG 8000, PrY–dextran 70 (0–50 μM dye), 50 mM sodium phosphate buffer (pH 7.9). Temperature, 22°C. □, n = 1.3; ■, n = 2.3; ○, n = 5.3; and ●, n = 8.3.

tively more PrY–dextran was needed to obtain saturation. As the ionic strength may influence the ligand–enzyme interaction, the effect of the concentration of phosphate buffer on the partitioning of the enzymes was investigated. On decreasing the concentration of phosphate buffer using saturating amounts of PrY–dextran (n = 5.3),

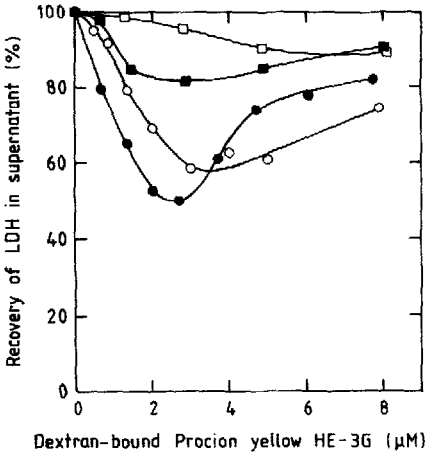

Fig. 4. Precipitation of LDH by PrY–dextran in the presence of PEG. LDH (1 kU l^{-1}) was incubated at 22°C with 80 g l^{-1} PEG containing 50 mM sodium phosphate buffer (pH 7.9) for 5 min and then centrifuged for 2 min at 8000 g. Symbols as in Fig. 3.

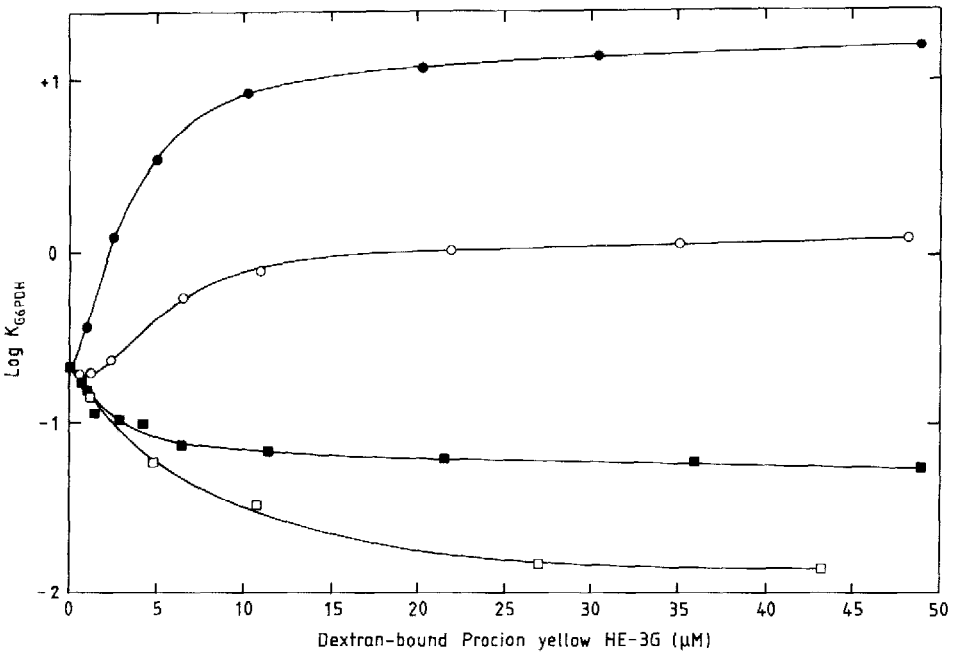

Fig. 5. Partitioning of glucose-6-phosphate dehydrogenase (7.7 kU l^{-1}) in systems containing increasing concentrations of PrY–dextran. Systems and symbols as in Fig. 3.

the maximal affinity partitioning effect (change in log K) first increased and then strongly decreased (Fig. 6). The maximal extraction effect was obtained in the sodium phosphate buffer (pH 7.5) concentration range 5–10 mM.

Binding of PrY–dextran to enzymes

The binding constants of dextran-bound dye to the three enzymes PGK, G6PDH and LDH were determined relative to the binding of their coenzymic nucleotides (ATP, NADP and NADH, respectively) (Table V). The influence of increasing number of dye ligands on the binding strength was moderate for PGK (one binding site), stronger for G6PDH (two binding sites) and very strong for LDH (four binding sites). Free (deactivated) Procion Yellow HE-3G showed a more than 10-fold stronger binding to the enzymes than dextran-bound dye (n = 1.3) or PEG-bound dye (n = 1.0).

The strong increase in the association constant of LDH with increasing number of dye ligands (Table V) shows that a chelating effect is involved. Although the ligands within the dextran molecule are randomly distributed, only a fraction might be localized in such way that effective chelating binding will occur. The reduction of binding strength when the dye is linked to a polymer is probably an effect of a negative interaction between the enzyme and polymer.

The extraction curves for LDH (Fig. 3) show half-saturation values of 3–4 μM dextran-bound dye in the ligand-dense phase (corresponding to 2–3 μM dye in the total system). These values are close to the calculated values (0.04–4 μM) obtained

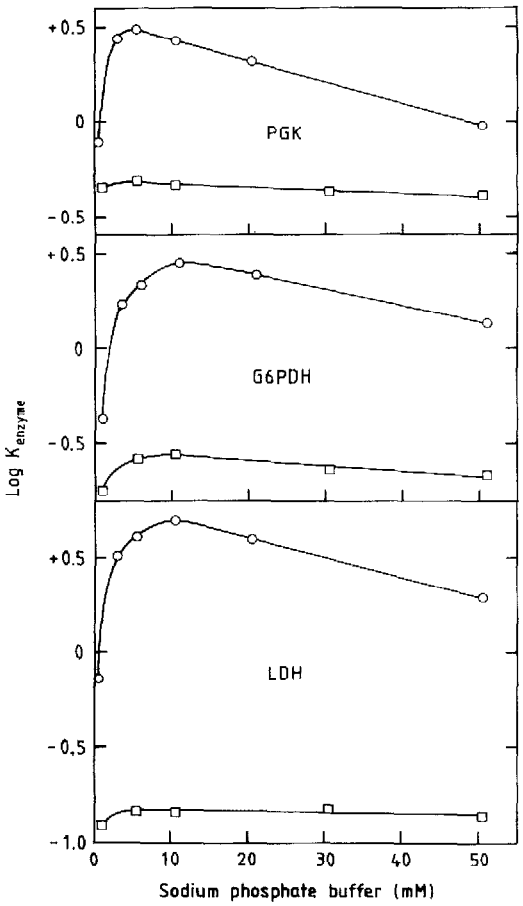

Fig. 6. Effect of concentration of sodium phosphate buffer (pH 7.5) on the partitioning of PGK (3.6 kU l^{-1}), G6PDH (7.7 kU l^{-1}) and LDH (12.5 kU l^{-1}). System composition: 8% (w/w) dextran 70, 4.5% (w/w) PEG 8000 with (○) or without (□) 30 μM dextran-bound Procion Yellow HE-3G (PrY–dextran 70, n = 5.3) and sodium phosphate buffer. Temperature, 22°C.

from the association constants for the dyes via Table IV and by using K_{diss} = 10 μM for the LDH–NADH dissociation constant[32]. A good correlation is also obtained for G6PDH, with a half-saturation value of 6–10 μM dye (in the ligand-rich phase) compared with the calculated K_{diss} = 3–25 μM (from K_{diss} = 50 μM for NADP[32]). However, no clear indication of steeper extraction profiles at higher n values can be seen. For LDH the assumed effective extraction predicted with highly substituted PrY–dextran might be hidden by the precipitating effect. Also, the presence of isoenzymes may effect the shape of the curve if they respond differently in the binding of PrY–dextran. The dissociation constant for PGK–PrY–dextran was found to be 5–20 μM (from K_{diss} = 110 μM for ATP)[32]. Extraction curves for PGK have not been determined.

TABLE V

ASSOCIATION CONSTANTS ($K_{ass, rel}$) OF POLYMER-BOUND AND FREE PROCION YELLOW HE-3G TO ENZYMES RELATIVE TO NUCLEOTIDE

PGK was measured in 54 m*M* triethanolamine–HCl (pH 7.6) and 0.27 m*M* ATP. G6PDH was measured in the same buffer but with 0.36 m*M* NADP. LDH was measured in 25 m*M* sodium phosphate buffer (pH 7.5) and 0.15 m*M* NADH. Temperature, 22°C.

Dye-containg substance	$K_{ass, rel}$		
	PGK	*G6PDH*	*LDH*
PrY–dextran 70 (n = 1.3)	5.4	2.0	2.9
PrY–dextran 70 (n = 2.3)	8.3	3.0	6.2
PrY–dextran 70 (n = 5.3)	11	6.3	66
PrY–dextran 70 (n = 8.3)	18	17	308
PrY–PEG 8000 (n = 1.0)	6.8	2.7	21
Free PrY	98	31	239

Relationship between partitioning of PrY–dextran and enzymes

The partitioning of PrY–dextran of various degrees of substitution and the enzymes PGK, G6PDH and LDH (without or with a saturating amount of dextran-bound Procion Yellow HE-3G) was determined in systems containing 10 m*M* sodium phosphate buffer (Table VI). The values obtained were compared with the general model for affinity partitioning according to Flanagan and Barondes[3]. This states that the partition coefficient of a protein, K, in excess of ligand polymer is related to the partition coefficient in the absence of the ligand, K_0, and the partition coefficient of the ligand polymer, K_L. If the number of ligand polymer molecules bound per protein molecule is a and the dissociation constants in the top and bottom phases are D_T and D_B, respectively, then the theory yields

$$K = K_0(K_L D_B/D_T)^a \quad (1)$$

If the dissociation constants are assumed to be equal, then this expression can be simplified to

$$K = K_0 K_L^a \quad (2)$$

or, in logarithmic form

$$\log K = \log K_0 + a \log K_L \quad (3)$$

The experimental K values (Table V) of the three enzymes partitioned in systems with excess of PrY–dextran are close to the calculated values obtained when PrY–dextran and protein are in the molar ratio 1:1 (PGK, G6PDH) or 1.5:1 (LDH). This indicates that one dextran molecule (or 1–2 in the case of LDH) surrounds the enzyme molecule linked to it via one or several ligands. This is in good correlation with the molecular dimensions of enzymes and dextran while PGK, G6PDH and LDH are compact structures (calculated diameters of equivalent spheres 43, 63 and 70 Å, re-

TABLE VI

COMPARISON OF CALCULATED AND EXPERIMENTALLY DETERMINED PARTITION COEFFICIENTS FOR PGK, G6PDH AND LDH

The theoretical partition coefficients for the enzymes were calculated from their partition coefficient, K_0, in the ligand-free system and the partition coefficient of PrY dextran, K_L, via the equation $\log K = \log K_0 + a \log K_L$, where a is the assumed number of dextran molecules bound per enzyme molecule. System, 8.0% (w/w) dextran 70, 30 μM dextran-bound Procion Yellow HE-3G (0.02–0.15% PrY–dextran 70), 4.5% (w/w) PEG 8000, 10 mM sodium phosphate buffer (pH 7.5) and with or without PGK (3 U ml^{-1}), G6PDH (7 U ml^{-1}) or LDH (12 U ml^{-1}). Temperature 22°C.

Ligand/dextran molar ratio	*Log K*							
	Pr–dextran, exptl.	*PGK*		*G6PDH*		*LDH*		
		Calc. (a = 1)	*Exptl.*	*Calc. (a = 1)*	*Exptl.*	*Calc. (a = 1)*	*Calc. (a = 2)*	*Exptl.*
0	–		−0.32	–	−0.56	–	–	−0.84
1.3	−0.47	−0.79	−0.91	−1.03	−1.41	−1.31	−1.78	−1.41
2.3	−0.12	−0.44	−0.43	−0.68	−0.81	−0.96	−1.08	−0.83
5.3	0.84	0.52	0.43	0.28	0.44	0.00	0.84	0.70
8.3	1.62	1.30	1.11	1.06	0.97	0.78	2.40	1.59

spectively). The length of the dextran molecule is 700–1000 Å. The latter can therefore effectively cover the surface of an enzyme molecule.

CONCLUSIONS

By introducing ligands in the dextran molecule its partitioning in an aqueous two-phase system is altered. If the ligand carries ionic groups the partition can be directed by including various salts in the system. The partitioning of ligand-binding enzymes is, in turn, determined by the partitioning of ligand–dextran. Consequently, an enzyme can be extracted into either the lower or the upper phase just by using different salts.

The affinity partitioning effect (changes in log K) seems to indicate that 1–2 dextran molecules are linked to the enzyme under saturation conditions.

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