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AFFINITY PARTITIONING: A NEW APPROACH FOR STUDYING DYE—PROTEIN INTERACTIONS

GERHARD KOPPERSCHLÄGER* and GERD BIRKENMEIER

Institute of Physiological Chemistry, Karl-Marx-University, Liebigstrasse 16, DDR-7010 Leipzig (G.D.R.)

SUMMARY

Affinity partitioning in an aqueous two-phase system composed of dextran and dye-liganded polyethylene glycol was applied to the investigation of the affinity of phosphofructokinase and glucose-6-phosphate dehydrogenase from baker's yeast, as well as of albumin and prealbumin from human serum to diverse reactive dyes. From the change in the partition coefficient K of the proteins in the two-phase system in the presence and in the absence of the dye-liganded polymer, expressed as $\Delta \log K$, quantitative data for the maximal extraction power and for the affinity of the proteins to various reactive dyes were obtained. The affinity partitioning effect on prealbumin is markedly increased by an excess of monomeric albumin. This points to an interaction of the two proteins in the presence of the dye, Remazol Yellow GGL. The competitive effect of various natural ligands on binding reactive dyes to proteins can be investigated by means of affinity phase partitioning as demonstrated on phosphofructokinase and prealbumin.

INTRODUCTION

The specific or group-specific affinity of certain biological macromolecules to natural and artificial ligands and the partition of these macromolecules between two non-miscible aqueous phases are combined in a selective extraction method called affinity partitioning. The two phases are obtained by dissolving two polymers in water. Several pairs of polymers can be used [1] but the most popular system is the one containing dextran and polyethylene glycol (PEG). A large number of variables allows the design of a system in which most proteins are concentrated in one phase containing mainly one kind of polymer [1, 2]. The selective extraction is due to covalent binding of a suitable ligand with high affinity for the target macromolecule to the polymer in the opposite phase, resulting in a significant change in the partition of the ligand-binding

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macromolecule. This alteration can be quantified by its corresponding partition coefficient, K [3].

The applicability of triazine dyes as group-specific ligands has been proved for the affinity partitioning of enzymes and other proteins by several groups [4–9]. There are numerous advantages for the employment of triazine dyes: (1) the high reactivity with either dextran or PEG, forming covalent links between the dye and the polymers; (2) the sufficient specificity of certain dyes to enzymes and other proteins and its reversibility in forming dye–protein complexes; and (3) the low cost of the pseudoligands when employing this method for large-scale separation.

EXPERIMENTAL

Dextrans [molecular weight (M_r) 70 000 and 500 000] were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) or from VEB Serumwerke Bernburg (Bernburg, G.D.R.). PEG 6000 (M_r 6000–7500) were from Union Carbide (New York, NY, U.S.A.) or from Serva, Feinbiochemica (Heidelberg, F.R.G.). The dyes were obtained from ICI, Organics Division (Blackley, Manchester, U.K.). Remazol Yellow GGL was supplied by Hoechst (Frankfurt, F.R.G.).

The two-phase systems (2 or 4 g, respectively) were prepared by weighing from aqueous stock solutions of PEG (40%, w/w), dextran (20%, w/w) and a stock buffer of 0.5 M sodium phosphate (pH 7.0) and various additives as indicated in the legends of the figures. The samples were cooled to 0°C and, after addition of a small volume of the respective protein solution, gently shaken for 15 s. Upon standing for ca. 5 min, the tube contents were mixed again and centrifuged at 3000 g for 2 min at 0°C. The partition coefficient, K, is defined as the ratio of the concentration of protein in the upper and lower phase.

Enzyme concentrations were determined by measuring the activity expressed in nkat (1 nkat = 1 nmol/s); prealbumin and albumin were determined by rocket electroimmunodiffusion. The dye–PEG derivatives were prepared according to Johansson [3], without prefractionation of the dye stuffs.

RESULTS AND DISCUSSION

In Fig. 1, a typical composition of a two-phase system for affinity partitioning is presented. In the absence of dye–PEG, more than 90% of the enzyme phosphofructokinase is concentrated in the lower dextran-rich phase. By replacing increasing amounts of the PEG by Cibacron Blue F3G-A–PEG (Cb–PEG), more and more of the enzyme is transferred into the upper phase. Thus, the partition coefficient K is increased, approaching a limiting K value of ca. 50. As shown in Fig. 2, the change in $\Delta \log K$, i.e. the difference in the logarithm of the K values with and without dye-liganded polymer, with the dye–polymer concentration follows, in this case, as well as in many other examples, a saturation curve from which two parameters can be derived: the maximum of $\Delta \log K$ and the half-saturation point $0.5 \times \log K_{max}$ (see inset of Fig. 2).

A number of enzymes and other proteins have been screened by this

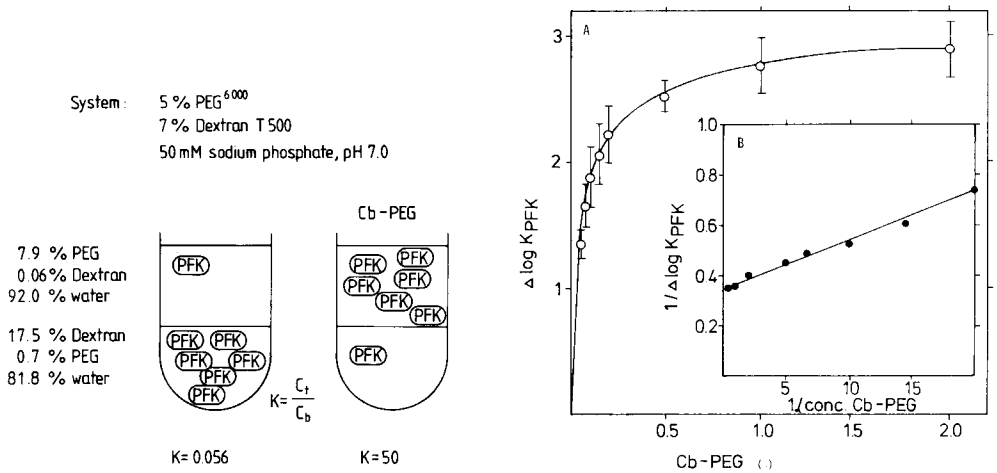

Fig. 1. Composition of a two-phase system and partition of phosphofructokinase.

Fig. 2. Change in the partition coefficient of phosphofructokinase as a function of Cb-PEG concentration. (A) Enzyme (16 nkat) was partitioned in a 4-g system containing 5% PEG 6000 and 7% dextran T 500 with different amounts of Cb—PEG, 50 m*M* sodium phosphate buffer (pH 7.0), 0.5 m*M* EDTA and 5 m*M* 2-mercaptoethanol. (B) Reciprocal plot of the data in A.

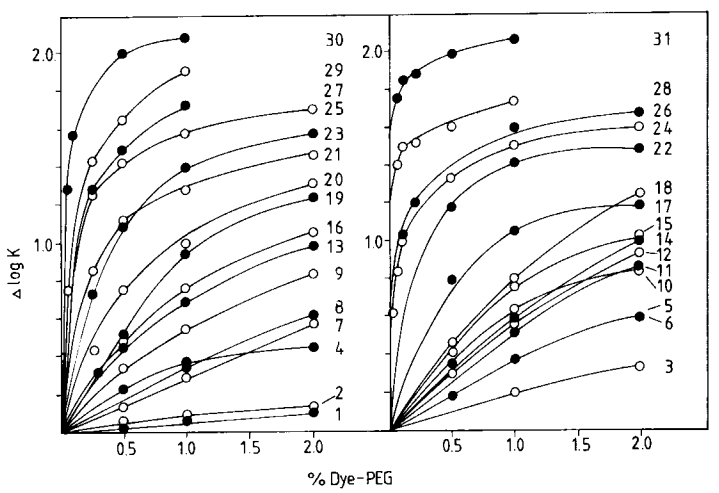

Fig. 3. Change in the partition coefficient of glucose-6-phosphate dehydrogenase with the concentration of diverse dye—PEG conjugates. The system (4 g) contains 5% PEG 6000 with different proportions of dye—PEG and 7.5% dextran T 500 or dextran M 70. The percentages in the figure refer to the portions of total polyethylene glycol that are replaced by dye-liganded PEG. Dyes are numbered as follows: (1) Procion Orange MX-G, (2) Procion Red H-3B, (3) Procion Yellow MX-4G, (4) Procion Red MX-8B, (5) Procion Blue MX-7RX, (6) Procion Red MX-5B, (7) Cibacron Brilliant Blue BRP, (8) Procion Scarlet H-RN, (9) Procion Yellow H-3R, (10) Procion Red P-3BN, (11) Procion Blue 5-HR, (12), Procion Red MX-G, (13) Procion Blue MX-R, (14) Procion Yellow MX-R, (15) Procion Blue SP-3R, (16) Procion Scarlet MX-G, (17) Procion Blue HE-RD, (18) Procion Yellow MX-GR, (19) Cibacron Blue F3G-A, (20) Procion Yellow HE-4R, (21) Procion Blue MX-3G, (22) Procion Brown H-5BR, (23) Procion Red HE-7B, (24) Procion Brown MX-5BR, (25) Procion Brown HE-G, (26) Procion Turquoise H-A, (27) Procion Red HE-3B, (28) Procion Yellow HE-3G, (29) Procion Green H-4G, (30) Procion Navy H-ER, (31) Procion Blue MX-G.

procedure and selective and subtle differences in the interaction of proteins and various dye stuffs have been found [6, 7]. An example for one enzyme is given in Fig. 3, in which yeast glucose-6-phosphate dehydrogenase was tested with 31 different triazine dyes covalently bound to PEG [10].

Of particular interest was the study of the interaction of human prealbumin and albumin with the dye Remazol Yellow GGL by means of affinity partitioning [11]. As seen in Fig. 4, both proteins are partitioned in a Remazol Yellow GGL—PEG-containing system yielding roughly the same value of $\Delta \log K_{max}$. Significant differences between the two proteins, however, exist in their relative affinities expressed as $0.5 \times \log K_{max}$, which are 45 and 110 μM for prealbumin and albumin, respectively. When albumin is added to the prealbumin-containing system, the transfer of the latter protein to the dye—polymer phase increased significantly without changing the relative affinity. The promoting action of albumin on the partition of prealbumin

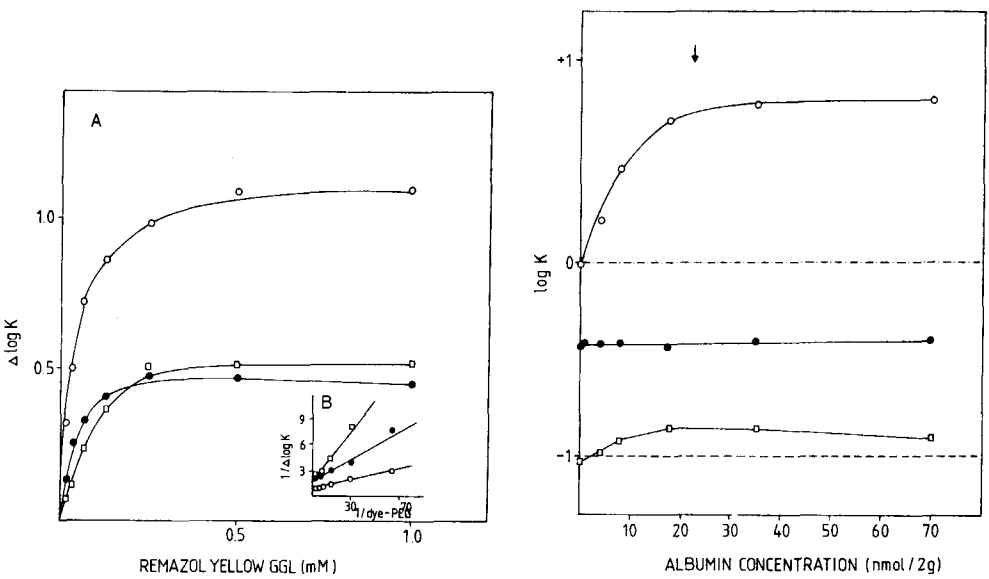

Fig. 4. Variation in the partition coefficient of prealbumin and albumin with increasing Remazol Yellow GGL-PEG concentration. (A) Systems of 2 g are composed of 10% dextran, 7.5% PEG containing different portions of the dye—PEG, 10 mM sodium phosphate buffer (pH 7.0) and protein. The log K values of prealbumin and albumin in the absence of dye—PEG are —0.39 and —1.37, respectively. The concentration of 1 mM dye—PEG in the upper phase corresponds to a total replacement of 3.2% PEG by dye—PEG. Proteins are: (□) albumin (4.3 μM), (•) prealbumin (1.6 μM), (○) prealbumin (1.6 μM) plus albumin (13.1 μM). (B) Reciprocal plot of the data in A.

Fig. 5. Affinity partition titration of prealbumin with increasing albumin concentration. Experimental conditions as in Fig. 4. (○) Prealbumin (3.2 nmol per 2 g system) in the presence of 1.6% Remazol Yellow GGL—PEG and increasing albumin concentration. (•) Prealbumin at increasing albumin concentration in the absence of dye—PEG. (□) Albumin at increasing concentration in the presence of 3.2 nmol prealbumin per 2 g system and 1.6% Remazol Yellow GGL—PEG. (- - - -) Partition of albumin alone in the presence of 1.6% Remazol Yellow GGL—PEG. (— • — • — •) Partition of prealbumin alone in the presence of 1.6% Remazol Yellow GGL—PEG. The arrow indicates the point of equivalent molar concentration of prealbumin and albumin in the upper phase.

seems to be specific, because albumin-depleted plasma was found to be without effect. Assuming an interaction between albumin and prealbumin, this would occur only in the presence of the dye, otherwise a change in the partition coefficient of prealbumin would also have been noticed in the absence of the dye.

The titration of the complex formation between prealbumin and albumin in the presence of Remazol Yellow GGL—PEG was carried out in a two-phase system (Fig. 5). With increasing concentration of albumin, the log K of prealbumin increases. A maximum value is attained when the concentration ratio of prealbumin to albumin in the upper phase is 1:1, corresponding to an overall concentration ratio of the system of ca. 1:7 (arrow in Fig. 5).

In order to study the influence of various effectors on dye—protein interaction, the method of affinity phase partitioning has been applied successfully. There are two possible approaches: determination of the changes in $\Delta \log K$ either with a constant level of effector and with varying concentrations of dye—PEG, or vice versa.

In Fig. 6, the influence of diverse nucleotides on the partition of phosphofructokinase at increasing Cb—PEG concentrations in the system is summarized. While AMP was found to be without any effect on partition of the enzyme, ITP-Mg^{2+}, ADP-Mg^{2+} and ATP-Mg^{2+} increasingly compete with the binding of Cibacron Blue. The maximum $\Delta \log K$ value is nearly the same for all systems (intercept with the ordinate) and the apparent values of Cb—PEG

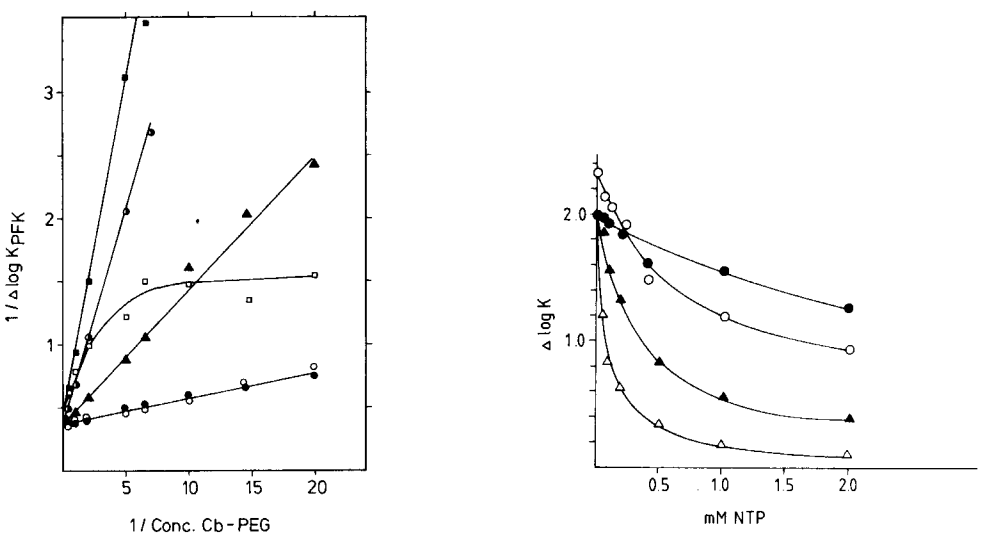

Fig. 6. Double-reciprocal plots of $\Delta \log K$ of phosphofructokinase and the concentration of Cb—PEG as a function of various effectors. Systems as described in Fig. 2 were used. The concentrations of the added effectors were: 1 mM and for $MgCl_2$ 2.5 mM. (•) Without effector, (○) 1 mM AMP, (▲) 1 mM ITP-Mg^{2+}, (□) 1 mM ATP, (◑) 1 mM ADP-Mg^{2+}, (■) 1 mM ATP-Mg^{2+}.

Fig. 7. Dependence of the $\Delta \log K$ of phosphofructokinase on the concentration of ATP and ITP. Systems as described in Fig. 2, containing 0 or 0.1 mM Cb—PEG, were used. Increasing amounts of the nucleotide triphosphates were added. (▲) ATP, (•) ITP, (△) ATP + 4 mM $MgCl_2$, (○) ITP + 4 mM $MgCl_2$.

concentration for half-saturation of the curve are: no effector or AMP 3.9 μM, ITP-Mg^{2+} 19.6 μM, ADP-Mg^{2+} 60 μM and ATP-Mg^{2+} 83 μM. The irregularity in competing effect of free ATP at low concentration of Cb–PEG allows us to suppose that the dye can occupy different ATP-binding sites. This was more evident in systems where the dye–PEG concentration was kept constant and the effector concentration was increased (Fig. 7). As shown, the competitive effect of the substrate ATP-Mg^{2+} is clearly recognizable, whereas free ATP in increasing amounts is less effective. The remaining $\Delta \log K$ value of the system could not be reduced further when the free ATP concentration was increased to 5 mM (not shown). It should be stressed that free ATP as well as ATP-Mg^{2+} are known as effective inhibitors to the enzyme [12]. ITP-Mg^{2+}, which is also a substrate of the enzyme but not an inhibitor causes a moderate effect in changing the $\Delta \log K$. However, the influence of free ITP on changing the partition of the enzyme is negligibly small.

From these results, the conclusion can be drawn that at least two different binding sites of the phosphofructokinase for ATP are involved in the binding of Cibacronblue, which was also suggested from earlier findings [9].

The competitive effect of different nucleotides on binding triazine dyes to lactate dehydrogenase, pyruvate kinase and glucose 6-phosphate dehydrogenase was also investigated by means of affinity phase partition. These results are summarized in refs. 6 and 10.

In the same line, it was of interest to study the influence of the natural ligands of prealbumin and albumin, i.e. the hormones thyroxine (T_4) and 3,3′,5-triiodothyronine (T_3), on the Remazol Yellow GGL–protein interaction. In Fig. 8, a strong competitive effect of T_4 and a moderate influence of T_3 on Remazol Yellow GGL–prealbumin binding is demonstrated, indicating that the

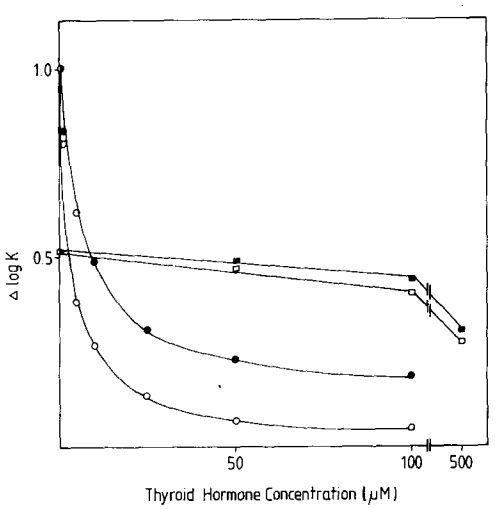

Fig. 8. Variation in the $\Delta \log K$ of prealbumin and albumin with concentration of thyroid hormones. Systems (2 g) contained 10% dextran, 7.5% PEG, 10 mM sodium phosphate buffer (pH 7.25), and increasing hormone concentration. (○, □) T_4 and (●, ■) T_3 were dissolved in 0.01 M sodium hydroxide, incubated with the proteins for 15 min at 22° C and then added to the system. (○, ●) Prealbumin (1.6 μM) in the presence of 30 μM albumin; (□, ■) albumin (30 μM).

dye binds closely or directly to the hormone-binding sites of T_4 and probably of T_3. The lower efficiency of T_3 in comparison to T_4 in competing with the dye parallels its lower affinity to the protein [13].

The competition of T_4 and T_3 on Remazol Yellow GGL—albumin binding, on the other hand, is negligible. Different binding sites possibly exist for the dye and for the hormones, respectively.

According to Flanagan and Barondes [14], the maximum change in the partition of a protein $\Delta \log K_{max}$ measured at the dye—PEG saturation point is assumed to be equal to the number of binding sites in the protein multiplied by the logarithm of the partition coefficient of the dye—PEG ($\log K_{dye-PEG}$) in the absence of proteins.

Assuming that the dissociation constants for the protein—dye—PEG complex are equal in the upper and in the lower phase, eqn. 1 results:

$$\Delta \log K_{max} = n \log K_{dye-PEG} \quad (1)$$

where n denotes the number of binding sites per protein molecule. In our experiments, the equation yields an apparent number of binding sites for phosphofructokinase, prealbumin and albumin, as given in Table I. The values are in contrast to the total number of binding sites of the dye and the natural ligands estimated directly.

TABLE I

COMPARISON OF NUMBER OF BINDING SITES FOR DYE—PROTEIN COMPLEXES OBTAINED BY AFFINITY PHASE PARTITIONING AND OTHER METHODS

Protein—dye	Affinity phase partitioning (apparent)	Equilibrium dialysis (total)
Phosphofructokinase—Cibacron Blue F3G-A	2	32*
Prealbumin—Remazol Yellow GGL	0.37	4
Albumin—Remazol Yellow GGL	0.4	8
Albumin—Cibacron Blue F3G-A	1.2	2**

*Determined by sedimentation velocity analysis [16].
**According to ref. 17.

There are several possible explanations for these discrepancies: (1) steric hindrance of binding the dye—PEG molecules at all binding sites, caused by the bulky PEG; (2) restriction of binding further ligands when the first dye—PEG is bound (in the case of phosphofructokinase); (3) only a small fraction of the dye participates in binding as a result of dye—PEG stacking; (4) significant differences exist in the dissociation constants of the dye—protein complex in the upper and in the lower phase.

In spite of insufficient correlation with the theoretical concept of Flanagan and Barondes [14], affinity phase partitioning can be successfully applied for the study of protein—dye interaction, as demonstrated. Moreover, this principle has been elaborated also for simple and effective extractions of enzymes and other proteins from different biological sources [4, 5, 15].

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