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Affinity partitioning of erythrocytic phosphofructokinase in aqueous two-phase systems containing poly(ethylene glycol)-bound Cibacron Blue

Influence of pH, ionic strength and substrates/effectors

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ABSTRACT

Phosphofructokinase (PFK) from rat eythrocyte haemolysates has a high affinity for Cibacron Blue F3G-A covalently bound to poly(ethylene glycol) (PEG–Cb) and thus the enzyme can be extracted into the top phase of poly(ethylene glycol)–dextran aqueous two-phase systems containing PEG–Cb. The pH, ionic strength and presence of substrates/effectors affect to different extents the affinity of the enzyme for PEG–Cb and the number of PEG–Cb molecules attached per molecule of PFK (the latter probably reflecting, at saturation, the influence on the aggregation state of the enzyme) and thus influence the yield of enzyme recovered in the top phase. Increasing the pH from 6 to 7 and then to 8 leads to a higher yield of PFK in the top phase. A change in pH from 6 to 7 and 8 results in an increased number of PEG–Cb molecules attached per molecule of enzyme while the affinity of PFK for PEG–Cb shows a minimum at pH 7. The ionic strength in the range 0.017–0.164 has less influence on the partitioning of PFK. The presence of substrates or effectors of the enzyme in general reduces the recovery of PFK in the top phase. Fructose 6-phosphate increases the number of PEG–Cb molecules attached but greatly reduces the affinity of PFK for PEG–Cb. In contrast, AMP slightly reduces the number of PEG–Cb molecules attached and the affinity of PFK for PEG–Cb. ATP and ATP–Mg^{2+} compete with PEG–Cb for the same binding sites in PFK. The best extraction of the enzyme is achieved at pH 8 and ionic strength 0.045: more than 95% of the enzyme is recovered in the top phase with a specific activity ten times higher.

INTRODUCTION

Affinity partitioning refers to extraction processes where one or several ligands selectively influence the partition of solutes (*e.g.* proteins) within aqueous two-phase systems of poly(ethylene glycol) (PEG) and dextran [1–3]. In principle, the ligand is immobilized to one of the polymers and thus restricted to one of the phases of the biphasic system. This methodology, which combines the simplicity and sensitivity of partitioning in aqueous two-phase systems with the specificity of affinity methods, has been extensively applied with two different purposes: first, to isolate a selected protein from crude extracts (yeast, animal cells, etc.) [4–6], and second, to recognize and characterize ligand–macromolecule interactions [7,8].

Triazine dyes covalently linked to PEG have been the ligands most extensively used in affinity partitioning and enzymes, especially among the groups of kinases and dehydrogenases, the target proteins [9–13]. The partition coefficient of the PEG–ligand is usually extremely high and therefore a great change in the partitioning of the protein is expected

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on attachment of only one or two molecules of the ligand [14]. A number of parameters have a pronounced effect on the partitioning of proteins in the affinity biphasic systems. In general, the partition coefficient (K) of the protein approaches a saturation value with increasing concentration of polymer-bound ligand; an increase in the polymer concentration of the system gives rise to more extreme K for the polymer-bound ligand and therefore larger increases in K value for the target protein; a lower pH value enhances the binding of PEG–triazine dyes to most proteins, thus reducing the specificity [15,16]. Other parameters such as salt and salt concentration, temperature, molecular weight of the polymers, free ligands and bulk proteins have different effects on different proteins [9,15,16].

The molecular mechanism of affinity partitioning is, however, not completely understood. The current theory developed by Flanagan and Barondes [17] predicts a linear relationship between the increment in log K of the protein produced by the affinity ligand (Δ log K) and the number of PEG–ligand molecules attached per molecule of protein. However, in most instances the total number of binding sites obtained by other methods are higher than the values resulting from partition experiments and, therefore, the attempts to correlate the Δ log K_{max} (under saturating conditions) with the number of actual binding sites failed [7,8,12].

In an attempt to optimize the extraction of phosphofructokinase (E.C. 2.7.1.11) (PFK) from rat erythrocyte haemolysates by affinity partitioning, the influence of several experimental conditions, including pH, ionic strength and presence of substrates/effectors of the enzyme, were studied. PEG covalently bound to Cibacron Blue F3G-A (PEG–Cb) was used because of the strong affinity shown by PFK towards this triazine dye ligand. The enzyme source was a PFK-enriched fraction obtained by selective precipitation with PEG [18]. We have previously shown that the extraction of PFK from rat erythrocytes by using affinity partitioning with PEG–Cb is greatly enhanced when a PFK-enriched fraction is used instead of the whole haemolysate [13]. The effects of the experimental conditions on the partitioning of PFK were analysed in the light of the current theory, in order to obtain an insight into the mechanism of the affinity partitioning.

EXPERIMENTAL

Chemicals

Polyethylene glycol (PEG-6000, MW 6000–7500) and Cibacron Blue F3G-A were purchased from Serva (Heidelberg, Germany), dextran T-500 (MW 500 000) from Pharmacia (Uppsala, Sweden), nucleotides (sodium salts of ATP and AMP), dithioerythritol, glucose 6-phosphate, fructose 6-phosphate (F6P) and coupling enzymes from Boehringer (Mannheim, Germany), EDTA (disodium salt) from Sigma (St. Louis, MO, USA) and Tris, 2-mercaptoethanol and all other chemicals (analytical-reagent grade) from Merck (Darmstadt, Germany).

Preparation of haemolysate

Anaesthetized male Wistar rats weighing 180–200 g were decapitated and whole blood was collected in heparinized tubes and centrifuged (400 *g*, 10 min). Red cells were washed three times with 0.15 *M* sodium chloride solution and freed from leucocytes and platelets by removing the top layer of cells after centrifugation. Subsequently, the red cells were lysed by mixing with an equal volume of a hypotonic solution consisting of 2.7 m*M* EDTA and 0.7 m*M* 2-mercaptoethanol (pH 7.3). The mixture was frozen and thawed twice and finally centrifuged (15 000 *g*, 30 min) to remove stroma and non-solubilized haemoglobin. All these steps were carried out at 4°C.

Preparation of phosphofructokinase-enriched fraction

A PFK-enriched fraction was obtained from the erythrocyte haemolysate by precipitation with PEG according to Tejedor *et al.* [18]. In brief, one volume of haemolysate (total protein about 20 mg/ml) was mixed with one volume of a solution of 12% (w/w) PEG in 96 m*M* potassium phosphate buffer (pH 6) containing 1 m*M* EDTA and 10 m*M* 2-mercaptoethanol. The mixture of PEG and haemolysate was kept in an ice-bath for 30 min and then centrifuged (5000 *g*, 10 min). The supernatant was discarded and the pellet containing 80% of the original PFK with a specific activity eight times higher was dissolved in 1 volume of 25 m*M* sodium phosphate buffer (pH 7) containing 0.5 m*M* EDTA and 5 m*M* 2-mercaptoethanol. This PFK extract was used immediately.

Preparation of biphasic systems

All the biphasic systems used in this study consisted of 5% PEG and 7.5% dextran and had a volume ratio of top to bottom phases close to unity. Portions of 1 g were made up in graduated tubes by weighing 0.1 g of PFK extract, appropriate amounts of distilled water, 40% (w/w) PEG, 20% (w/w) dextran (standardized by polarimetry) and stock phosphate buffer to produce the pH and ionic strength required (see legends to the figures). The substrates/effectors were introduced into the system by replacing the distilled water with appropriate stock solutions to provide the desired concentration (see legends to the figures).

All the components were mixed well by 30–40 inversions and then the systems were left to settle at 4°C until complete separation of the phases was achieved. Aliquots from top and bottom phases were then analysed for protein concentration and enzymatic activity.

The affinity systems were prepared as above but using a 40% (w/w) stock solution of PEG in which increasing amounts of PEG had been replaced with PEG–Cb. The biphasic systems thus obtained had 5% total PEG (PEG + PEG–Cb) but increasing proportions of PEG–Cb from 0.05 to 0.5% (% PEG–Cb in the system). The substituted polymer (PEG–Cb) was prepared as described by Delgado *et al.* [13].

The partition coefficient, K, is defined as the ratio between the enzyme activities in the top and bottom phases of the systems. The percentage of PFK in the top phase of the system was calculated from the partition coefficient and volume ratio values as described by Johansson [16]. The concentrations of polymers in the systems are always expressed as w/w.

Enzyme and total protein assays

PFK activity was measured by the linear decrease in NADH absorbance (340 nm) in a test mixture containing 100 mM Tris–HCl (pH 7.1), 0.3 mM EDTA, 3 mM magnesium chloride, 90 mM potassium chloride, 0.15 mM NADH, 1.5 mM dithioerythritol, 1 mM phosphate, 3 mM F6P, 9 mM glucose 6-phosphate, 1.5 mM ATP, 0.1 U/ml aldolase (E.C. 4.1.2.13), 6 U/ml triosephosphate isomerase (E.C. 5.3.1.1) and 0.5 U/ml glycerol-3-phosphate dehydrogenase (E.C. 1.1.1.8). The total protein concentration was measured by the Coomassie Brilliant Blue assay [19].

RESULTS

The partitioning of rat erythrocytic PFK in an aqueous two-phase system consisting of 5% PEG, 7.5% dextran and increasing concentrations of PEG–Cb was studied under different conditions of pH, ionic strength and effectors of the enzyme (Fig. 1). PFK has affinity for the PEG–Cb under all the conditions studied, as shown by the increased partition coefficient (expressed on a logarithmic scale) in the presence of this polymer compared with the control system (not containing PEG–Cb). In all instances, the partition coefficient increases progressively with increasing concentration of PEG–Cb in the biphasic system finally to reach a plateau (Fig. 1).

The influence of the pH in the range 6–8 is shown in Fig. 1A. In the control system the partition coefficient in PFK has values of 0.1, 0.07 and 0.05 ($\log K = -1.00$, -1.15 and -1.30) for pH values of 6, 7 and 8, respectively. In the affinity systems, increases in the pH from 6 to 7 and 8 lead to increases in the partition coefficient at all concentrations of PEG–Cb (Fig. 1A).

The influence of the ionic strength was studied in the range 0.017–0.3 (Fig. 1B). In the control system the partition coefficient varies from 0.03 ($\log K = -1.52$) to 0.15 ($\log K = -0.82$). In the affinity systems, similar partitioning behaviours are found for PFK in the ionic strength range 0.017–0.082 (Fig. 1B). An increase in the ionic strength to 0.164 leads to lower values for the partition coefficient at concentrations of PEG–Cb up to 0.3% whereas above this concentration the partition coefficients are similar to those obtained in the ionic strength range 0.017–0.082 (Fig. 1B). A further increase in the ionic strength to 0.3 leads to lower partition coefficients at all concentrations of PEG–Cb (Fig. 1B).

The influence of a constant concentration of some substrates and effectors of PFK on its partitioning behaviour is shown in Fig. 1C. The partition coefficient of PFK is reduced by the presence of any of the substrates or effectors in the affinity systems. AMP has only a slight influence on the saturation curve followed by F6P (Fig. 1C). In the

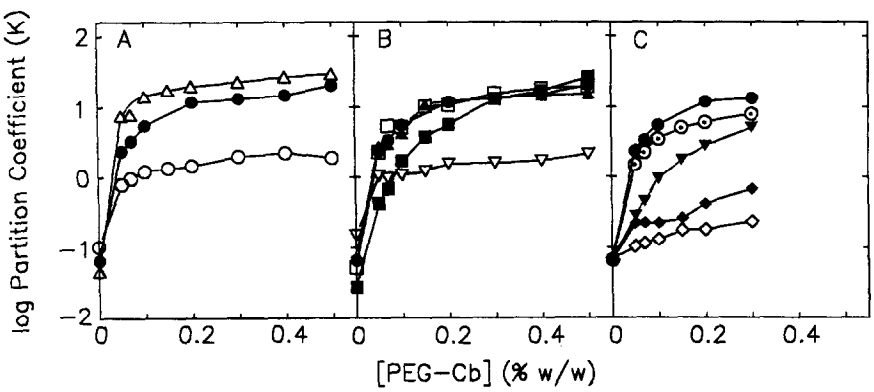

Fig. 1. Influence of increasing concentrations of PEG–Cb on the partitioning of erythrocytic PFK in a biphasic system consisting of 5% total PEG (PEG + PEG–Cb) and 7.5% dextran under the following experimental conditions: (A) (○) pH 6, (●) pH 7 and (△) pH 8 at a constant ionic strength of 0.045; (B) ionic strength (▲) 0.017, (●) 0.045, (□) 0.082, (■) 0.164 and (▽) 0.3 at pH 7; (C) (●) control, (⊙) 1 m*M* AMP, (▼) 1 m*M* F6P, (◆) 1 m*M* ATP and (◇) ATP–Mg^{2+} (1 m*M* ATP, 2.5 m*M* Mg^{2+}) at pH 7 and ionic strength 0.045. All the systems contain 0.25 m*M* EDTA, 2.5 m*M* 2-mercaptoethanol and sodium phosphate buffer as follows: to obtain a constant ionic strength of 0.045, 40.17 m*M*, 25 m*M* and 15.08 m*M* for pH 6, 7 and 8, respectively; to obtain a constant pH of 7, 9.1 m*M*, 46 m*M*, 92 m*M* and 169 m*M* for ionic strength 0.017, 0.045, 0.082, 0.164 and 0.3, respectively.

presence of ATP the saturation curve consists of two components (Fig. 1C). ATP–Mg^{2+} produces the greatest reduction in the PFK partition coefficient (Fig. 1C).

The maximum extraction of PFK in the top phase of the affinity biphasic systems takes place at pH 8 and ionic strength 0.045. The partition coefficient varies from 20 (log K = 1.3) in the biphasic system containing 0.2% PEG–Cb to 31 (log K = 1.5) in the system containing 0.5% PEG–Cb. These partition coefficients correspond to 95.24% and 96.88% of PFK extracted in the top phase, respectively. The higher the concentration of PEG–Cb the more viscous and hence more difficult to handle is the biphasic system, hence the extraction of PFK using the system containing 0.2% PEG–Cb might have advantages, especially when scaling up the system. The partition coefficient of the total proteins is of about 0.1, which leads to a purification factor of about 10 for PFK in the top phase.

The affinity partitioning effect promoted by PEG–Cb is quantitatively analysed in double-reciprocal plots of the increment in log K (Δ log K) (defined as the difference between log K in the presence and the absence of the ligand) *versus* the concentration of PEG–Cb (Fig. 2) in order to calculate the values for $[PEG–Cb]_{0.5}$ and Δ log K_{max}. The former corresponds to the concentration of PEG–Cb at which half of the available sites in the enzyme are occupied by the ligand, *i.e.* it measures the affinity. The latter measures the maximum change in the partition coefficient promoted by the ligand. The number of binding sites for the ligand is then calculated by the ratio between Δ log K_{max} and log K for the free ligand [7,16]. Log K for free PEG–Cb in the systems used in this study was in the range 0.3–0.6 with a mean value of 0.47. The number of binding sites thus obtained is given in Table I.

An increase in pH from 6 to 8 leads to a dramatic increase in Δ log K_{max} and hence the number of binding sites. The affinity of PFK for PEG–Cb has a minimum at pH 7 (shown by the increase in $[PEG–Cb]_{0.5}$) (Table I). Ionic strength in the range 0.017–0.164 has only a slight influence on Δ log K_{max} whereas the affinity of PFK for PEG–Cb decreases on increasing the ionic strength. This is especially marked when the ionic strength increases from 0.082 to 0.164 (Table I). When the ionic strength is further increased to 0.3, the double-reciprocal plot deviates considerably from a linear relationship (Fig. 2B) and therefore Δ log K_{max} and $[PEG–Cb]_{0.5}$ could not be calculated.

The presence of effectors or substrates of PFK influences the affinity and Δ log K_{max} to different extents (the control is at pH 7, ionic strength 0.045). F6P reduces drastically the affinity of PFK for PEG–Cb whereas Δ log K_{max} shows a maximum (Table I). In contrast, AMP produces only a slight

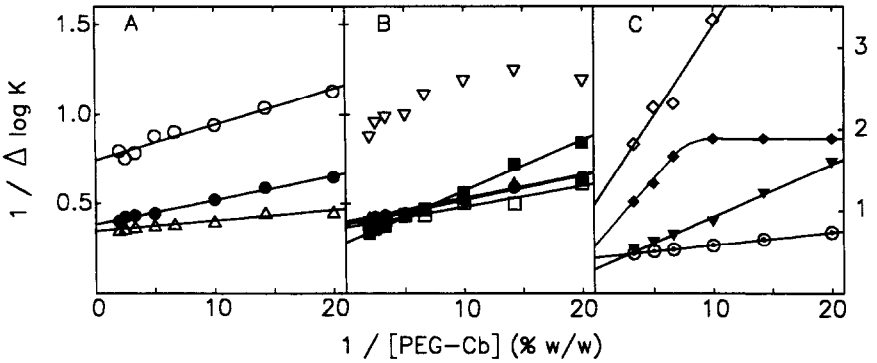

Fig. 2. Double-reciprocal plots of Δ log K (difference between log K in the presence and absence of PEG–Cb) *versus* concentration of PEG–Cb.

decrease in the affinity of PFK for PEG–Cb and has little influence on Δ log K_{max} (Table I).

In the presence of ATP two slopes are observed (Fig. 2C). One corresponds to a binding site which is not saturable by PEG–Cb ($[PEG–Cb]_{0.5}$ is infinite) and which accounts for 25% of the binding sites available (the total is the sum of binding sites for the two slopes). The other, accounting for 75% of the available binding sites, shows the largest reduction in the affinity of PFK for PEG–Cb under all the conditions tested (Table I). In the presence of ATP–Mg^{2+} both the affinity and Δ log K_{max} are considerably reduced (Table I).

In order to obtain an insight into the nature of the binding sites for PEG–Cb in erythrocytic PFK, affinity partitioning was studied at a single concentration of PEG–Cb (0.3%) and increasing concentrations of F6P or ATP (Fig. 3A). F6P produces a slight reduction in the log K which plateaus at a concentration of 1.5 mM. This behaviour of PFK could be expected in the presence of a non-competitor. However, increasing concentrations of ATP lead to a steady reduction in log K to approach the value for log K in the absence of PEG–Cb.

To analyse further the affinity partitioning effect, a plot of Δ^* log K (absolute value) *versus* the con-

TABLE I

AFFINITY PARTITIONING PARAMETERS FOR ERYTHROCYTIC PFK IN 5% PEG, 7.5% DEXTRAN TWO-PHASE SYSTEM CONTAINING PEG–Cb.

Maximum increment of the partitioning coefficient and relative binding affinity of PEG–Cb for erythrocytic PFK were calculated by linear regression of the double-reciprocal plots. The number of PEG–Cb binding sites was calculated from the simplified Flanagan and Barondes equation [17].

Experimental conditions	Δ log K_{max}	$[PEG–Cb]_{0.5}$ (%, w/w)	No binding sites
pH 6	1.3	0.025	2.8
pH 7	2.5	0.035	5.3
pH 8	2.8	0.015	6.0
Ionic strength 0.017	2.4	0.030	5.1
Ionic strength 0.045	2.5	0.035	5.3
Ionic strength 0.082	2.6	0.045	5.5
Ionic strength 0.164	3.0	0.100	6.4
1 mM F6P	3.3	0.200	7.0
1 mM AMP	2.3	0.050	4.9
1 mM ATP (1st slope)	0.5	–	1.1
1 mM ATP (2nd slope)	1.9	0.300	4.1
ATP–Mg^{2+}	0.9	0.200	1.9

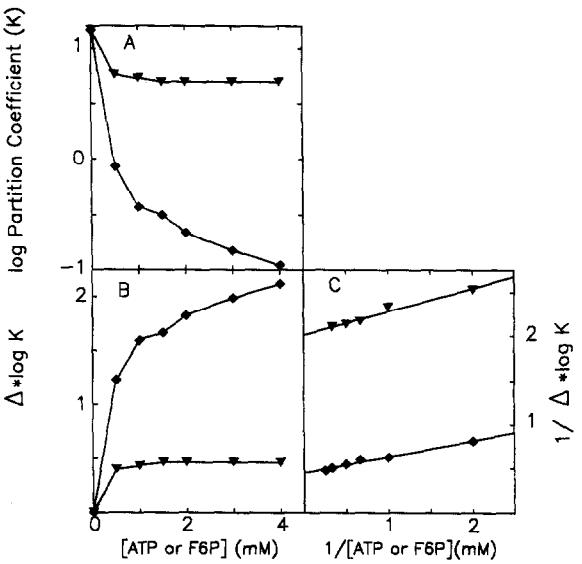

Fig. 3. (A) Influence of increasing concentrations of (▼) F6P and (◆) ATP on the partitioning of erythrocytic PFK in a biphasic system consisting of 5% total PEG (PEG + PEG–Cb), 7.5% dextran and 0.3% PEG–Cb at pH 7 and ionic strength 0.045. (B) Variation of Δ^* log K (difference between log K in the presence and absence of ATP or F6P) as a function of the concentration of F6P or ATP. (C) Double-reciprocal plots of the data in (B).

centration of F6P or ATP was constructed (Fig. 3B). Δ^* log K refers to the difference between log K in the presence and the absence of effector, both situations in the presence of 0.3% PEG–Cb. A double-reciprocal plot (Fig. 3C) provides the values for $[\text{effector}]_{0.5}$ and Δ^* log $K_{max} \cdot \Delta^*$ log K_{max} represents the maximum reduction that can be produced by the substrate in the affinity partitioning promoted by 0.3% PEG–Cb (with this concentration of PEG–Cb, pH 7 and ionic strength 0.045, Δ log K was near 2.5). In the presence of F6P, Δ^* log K_{max} is equal to 0.5 and $[\text{F6P}]_{0.5}$ is infinite. These results taken together reflect that F6P cannot prevent the binding of PEG–Cb to PFK. In the presence of ATP, $[\text{ATP}]_{0.5}$ is 0.53 mM and Δ^* log K_{max} is equal to 2.5.

DISCUSSION

Rat erythrocytic PFK shows a high affinity for the triazine dye Cibacron Blue, similar to that already reported for yeast PFK. This behaviour allows the use of affinity partitioning in PEG–dextran two-phase systems with PEG–Cb as affinity ligand for a partial but significant purification of this enzyme from haemolysates with a yield of about 95%. Affinity partitioning has proved to be useful for the purification of several enzymes from yeast, animal tissues and plants [1–6]. In most instances single extraction steps are used as the partition coefficient of the protein of interest is many times higher than that of the bulk proteins [4,6]. A common feature for all the examples is the high yield in the selected protein achieved at the end of the process, which provides a great advantage over other more classical methods. In addition, combination of affinity partitioning with counter-current distribution (multiple extraction steps) has led to the fractionation of complex mixtures into several of their components [10].

Under all conditions studied, the partition coefficient of PFK plateaus *versus* the concentration of PEG–Cb. Two main conclusions are drawn from this behaviour: (a) there is a maximum amount of enzyme that can be extracted into the top phase of the biphasic system under any of the conditions studied and (b) under the conditions where the plateau is reached below the highest value (obtained at pH 8), the maximum value reached for the partition coefficient does not represent saturation of the extraction capacity of the biphasic system.

The affinity partitioning effect depends on two critical factors: the affinity of the enzyme for PEG–Cb and the number of PEG–Cb molecules attached per molecule of enzyme. Ideally, to achieve maximum extraction of the target protein, experimental conditions have to be selected to provide maximum values for both of them. With erythrocytic PFK the best extraction conditions are pH 8 and ionic strength 0.045, where a high affinity of PEG–Cb for PFK is observed together with a high value of Δ log K_{max}.

The decreased binding of PEG–Cb to erythrocytic PFK at low pH seems to be a peculiar feature of this enzyme. It is well documented that with triazine dyes, a lower pH value enhances the binding to most enzymes [14]. This behaviour in turn leads to a reduction in the specificity of the extraction. Other exceptions to this effect of pH include some serum proteins [20].

The changes in the affinity of PFK for PEG–Cb probably reflect conformational changes of the

binding site and/or surroundings promoted by the different experimental conditions. Δ log K_{max} is related to the number of binding sites for PEG–Cb per molecule of protein. Hence an increase in Δ log K_{max} will be found on association of PFK subunits to form larger oligomers, whereas a decrease in Δ log K_{max} will take place on dissociation of the subunits. Analysis of the data in Table I in this light indicates the following: a decrease in pH from 7 to 6 produces dissociation of erythrocytic PFK whereas an increase in pH to 8 leads to association to larger oligomers. Such effects of pH on erythrocytic PFK have been reported previously [21]. Ionic strength in the range 0.017–0.164 has little effect on the PFK self-association equilibrium. AMP has no influence on the self-association equilibrium and F6P produces aggregation of the enzyme. The effect of ATP on the self-association equilibrium of PFK cannot be studied using PEG–Cb as the affinity ligand, as it binds to the same sites as ATP and therefore the enzyme cannot be saturated with ATP for it to produce an effect.

The results obtained in the presence of F6P support the concept that maximum extraction of the enzyme in the top phase should be obtained when the enzyme is more aggregated. This would be the case if no negative effect on the affinity of PFK for PEG–Cb were to be produced (this makes the concentration of PEG–Cb required to achieve Δ log K_{max} impracticable). The reduction in affinity produced by F6P has also been found in studies with yeast PFK [7,22]. That behaviour was interpreted in two different ways: (a) the binding of F6P causes steric hindrance of the complex formation with the dye or (b) the substrate lowers the affinity to the dye by shifting the allosteric equilibrium towards the R-state [22]. The difference in Δ log K_{max} for the control system and the system with F6P indicates that F6P does not compete with PEG-Cb for the same binding sites in PFK.

The presence of two components in the saturation curve with ATP might represent displacement of the ATP by increasing concentrations of PEG–Cb from two binding sites in the enzyme with different affinities for ATP. The complete displacement of PEG–Cb by ATP (Δ log K_{max} and Δ^* log K_{max} have the same absolute value) strongly supports competition of ATP and PEG–Cb for the same binding sites and, as a result, ATP can be used to strip off the PFK efficiently from the PEG–Cb-containing phase. The magnitude of the competition might also indicate that all the ATP available sites in PFK are occupied by PEG–Cb. If some of the ATP binding sites were unoccupied by PEG–Cb, then the reduction in the partition coefficient would have consisted of two phases: in the first phase (lag phase), the ATP binds to the free available sites and no effect (or little) is seen in the affinity partitioning effect promoted by PEG–Cb; in the second phase, ATP and PEG–Cb bind to the same sites in PFK, resulting in a considerable reduction in the affinity partitioning as the concentration of ATP is increased.

In conclusion, affinity partitioning is a powerful technique for the rapid isolation of proteins from crude extracts. Advantage can be taken of experimental conditions known to promote aggregation of the protein provided that the affinity of the protein for the ligand is not reduced.

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