A Conformational Transition Involved in Antagonistic Substrate Binding to the Allosteric Phosphofructokinase from *Escherichia coli*†

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ABSTRACT: The binding of fructose 6-phosphate, ATP or its nonhydrolyzable analogue adenylyl 5′-(β,γ-methylenediphosphonate), ADP, and phosphoenolpyruvate to *Escherichia coli* phosphofructokinase has been studied by changes in the protein fluorescence and/or equilibrium dialysis. The results lead to the following conclusions: (1) tetrameric phosphofructokinase can bind four ATP but only two fructose-6-phosphate, and this binding occurs without cooperativity; (2) only two conformational states, T and R, with respectively a high and a low fluorescence, seem accessible to phosphofructokinase, which exists as a mixture of one-third R and two-third T states in the absence of ligand; (3) the substrate fructose 6-phosphate and the allosteric activator ADP bind preferentially to the low-fluorescence R state, while the other substrate, ATP [or its nonhydrolyzable analogue adenylyl 5′-(β,γ-methylenediphosphonate)], and the allosteric inhibitor phosphoenolpyruvate bind to the high-fluorescence T state; (4) the binding of a given ligand is cooperative, with a Hill coefficient of 2, only when this binding is accompanied by a complete shift from one state to the other; for instance, the binding of the ATP analogue adenylyl 5′-(β,γ-methylenediphosphonate) to the T state is cooperative only in the presence of fructose 6-phosphate which favors the R state. This behavior is qualitatively consistent with a concerted transition, but quite different from that described earlier for phosphofructokinase from steady-state activity measurements (Blangy et al., 1968). This discrepancy suggests that the allosteric properties of phosphofructokinase are due in part to ligand binding and in part to the kinetics of the enzymatic reaction.

Phosphofructokinase (PFK)[1] catalyzes the reaction:

ATP + fructose 6-phosphate → ADP + fructose 1,6-bisphosphate

In *Escherichia coli*, the enzyme present as the major species is a tetramer of 4 identical subunits of 320 amino acids of known sequence (Hellinga & Evans, 1985) and three-dimensional structure (Shirakihara & Evans, 1988; Rypniewsky & Evans, 1989). This PFK is a regulatory enzyme subject to allosteric control. Its activity is increased by the purine nucleoside diphosphates ADP or GDP and inhibited by phosphoenolpyruvate (PEP). In addition, the saturation of PFK by its substrate fructose 6-phosphate (Fru-6P) is highly cooperative (Blangy et al., 1968). The steady-state kinetics of *E. coli* PFK are consistent with the concerted allosteric mechanism (Monod et al., 1965) in which two conformational states of the protein, R and T, are in equilibrium. The R and T states of PFK have the same affinity for ATP, but the apparent affinity for Fru-6P of the R state is 2000 times higher than that of the T state. In the absence of ligand, PFK is mainly in the T state with the fraction of the protein in the R state being less than 10^{-6} (Blangy et al., 1968). These states do not have the same affinity for the allosteric effectors: the activator ADP (or GDP) binds preferentially to the R state, and the inhibitor PEP binds to the T state. Thus, the activators ADP and GDP increase the affinity of PFK for Fru-6P by favoring the R state, while the inhibitor PEP decreases this affinity by favoring the T state (Blangy et al., 1968; Blangy, 1971).

Some recent results suggest, however, that a unique structural transition between the two states R and T cannot account for both the homotropic allosteric effects, i.e., the cooperativity of saturation by Fru-6P, and the heterotropic effects, i.e., the regulation by effectors, at the same time: (i) the three-dimensional structure of unliganded PFK resembles that of the R state (Rypniewsky & Evans, 1989), whereas the concerted model predicts that it should be in the T state (Blangy et al., 1968); (ii) in the absence of effector, the three-dimensional structure of the PFK from *E. coli* (Rypniewsky & Evans, 1989) is similar to that from *Bacillus stearothermophilus* (Evans et al., 1981), which is mostly in the R state since its saturation by Fru-6P is hyperbolic (Valdez et al., 1989); (iii) the pH-dependence of the properties of *E. coli* PFK suggests that part of the cooperativity observed in saturation by Fru-6P is of kinetic origin (Deville-Bonne et al., 1991a); (iv) some mutant PFKs have properties which are not easily accounted for by the concerted model (Lau & Fersht, 1987; Perutz, 1987; Berger & Evans, 1990; Serre et al., 1990).

In order to evaluate the binding and kinetic contributions to the overall cooperativity observed for the saturation of PFK by Fru-6P, we have directly measured the binding of both substrates to PFK by equilibrium dialysis and by fluorescence changes.

Materials and Methods

Chemicals. The auxiliary enzymes used in the coupled assay (aldolase, triosephosphate isomerase, glycerolphosphate dehydrogenase), GDP, PEP, and adenylyl 5′-(β,γ-methylenediphosphonate) (AMPPCP) are obtained from Boehringer.

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[1] Abbreviations: PFK, phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11); AMPPCP, adenylyl 5′-(β,γ-methylenediphosphonate); DTT, DL-dithiothreitol; Fru-6P, D-fructose 6-phosphate; PEP, phosphoenolpyruvate.

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ATP, dithiothreitol (DTT), Fru-6P, and NADH are from Sigma. [U-^{14}C]Fru-6P is obtained from CEN Saclay and [2,8-^{3}H]ATP from Amersham.

Purification of PFK. PFK is prepared from HE1 cells transformed with the plasmid pHL1 as previously reported (Deville-Bonne et al., 1989). The purified enzyme is stored as a 55% ammonium sulfate precipitate (Kotlarz & Buc, 1982) and is extensively dialyzed before use against three changes of 1.5-L total volume of 100 mM Tris-HCl buffer containing 1 mM magnesium acetate and 2 mM DTT, pH 8.2 (T buffer). Protein concentration is determined from the absorbance at 278 nm using an extinction coefficient of $\epsilon_{278} = 0.6$ cm^2·mg^{-1} (Kotlarz & Buc, 1977). Alternatively, protein concentration is measured according to Bradford (1976) using a factor of 0.6 to take into account the difference between PFK and the standard protein used, immunoglobulin.

Equilibrium Dialysis Experiments. Binding of [^{14}C]Fru-6P or [^{3}H]ATP to PFK is studied at 20 °C by equilibrium dialysis using Plexiglass dialysis microcells (50 μL in each compartment) equipped with Spectrapor 2 cellulose tubing membranes (Spectrum). For Fru-6P binding, PFK, at a concentration of 75–150 μM in subunits is incubated in T buffer supplemented with 10 mM magnesium acetate and 20–400 μM [^{14}C]Fru-6P with a specific radioactivity varying from 30 to 100 Bq/nmol. For ATP binding, PFK, at a concentration of 10 μM in subunits is incubated in T buffer with 10 mM magnesium acetate and 1–50 μM [^{3}H]ATP with a specific radioactivity varying from 2 to 40 Bq/pmol. Equilibration of the ligand between the two compartments is reached within 6 h at 20 °C under gentle rotation of the cells. Correction for ligand binding to membranes in unnecessary as the totality of radioactivity is recovered from both cells. It has been checked that PFK retains its full enzymatic activity after 6 h of dialysis.

Fluorescence Measurements. Steady-state fluorescence measurements are made in a Spex Fluorolog 2 equipped with a DM 1B spectroscopy laboratory coordinator. Intrinsic PFK fluorescence is measured at 340 nm upon excitation at 295 nm and is due to the unique tryptophan Trp-311. A decrease in fluorescence is observed upon Fru-6P addition and is related to the extent of Fru-6P binding (Berger & Evans, 1991). Fluorescence measurements are carried out in a buffer composed of 0.1 M Tris-HCl, pH 8.2, containing 1 mM magnesium acetate. The emitted intensity is proportional to PFK concentration up to 60 μg/mL, i.e., 1.7 μM in subunits, but the signal is slightly quenched by magnesium or phosphate ions. PFK solutions are titrated with Fru-6P delivered with an Eppendorf Varipette 4710. Fluorescence reading is corrected for dilution and for nonspecific fluorescence monitored in the presence of phosphate at the same concentration. The inner filter effect is also negligible as protein absorbance is always less than 0.04 at 295 nm. In order to compare equilibrium dialysis and fluorescence data, it has been checked that the fluorescence signal is attained almost instantaneously upon mixing and is constant over longer incubation times (6 h).

Data Analysis. Each binding experiment has been fitted to hyperbolic ligand–protein interaction and/or cooperative interactions by the Hill equation using the program Enzfitter (Leatherbarrow, 1987) or Mdfitt (M. Desmadril, personal communication). The whole sets of equilibrium dialysis and fluorescence data have been analyzed separately using the statistical procedure of Johnson and Frazier (1985).

Results

Equilibrium Dialysis Measurement of the Binding of ATP and Fru-6P to PFK. The interaction of PFK with one of its substrates, ATP or Fru-6P, was measured by equilibrium dialysis. No evidence of cooperative binding of ATP or Fru-6P to PFK is observed, and attempts to fit the data with the Hill equation lead to values of the cooperativity coefficient, n_H, lower than 1.2. The formation of the binary complex between PFK and ATP follows a hyperbolic saturation, with a dissociation constant of 10 μM and a stoichiometry of 3.7 ATP bound per tetrameric PFK (result not shown). So, the binding of ATP takes place at four independent sites, in agreement with previous results (Blangy, 1971). These sites are probably the four active sites identified by X-ray crystallography (Shirakihara & Evans, 1988).

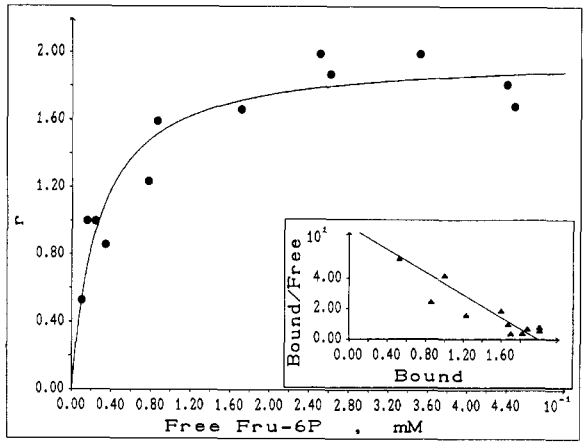

FIGURE 1: Binding of fructose 6-phosphate to PFK measured by equilibrium dialysis. PFK, 38 μM in tetramer, in 0.1 M Tris-HCl, pH 8.2, containing 10 mM magnesium acetate and 2 mM DTT was incubated with Fru-6P as described under Materials and Methods. The ratio r of bound Fru-6P per tetramer is plotted as a function of free [Fru-6P]. The line corresponds to the best hyperbolic fit for this set of data. Inset: Scatchard plot of the same data.

There is also formation of a binary complex between PFK and Fru-6P (Figure 1), which follows a hyperbolic saturation, with a dissociation constant of 32 μM (mean of five experiments). No significant difference in Fru-6P binding is found in the presence of 1 mM magnesium or 10 mM ammonium ions. In a previous study (Blangy, 1971), this binding of Fru-6P to PFK has been overlooked probably because it was expected at lower Fru-6P concentrations. The dissociation constant of 12 μM calculated previously from steady-state enzymatic activity (Blangy et al., 1968) is smaller than that measured here. Also, such a measurement was difficult due to the limited specific radioactivity of [^{14}C]Fru-6P and to the low-affinity constant of PFK for Fru-6P. The existence of a binary complex between PFK and Fru-6P has already been suggested by the marked stabilization afforded by Fru-6P alone against the denaturation of the protein induced by different agents: temperature (Blangy, 1971; Le Bras & Garel, 1985), solvent additives (Deville-Bonne et al., 1989; Le Bras et al., 1990; Teschner et al. 1990), or high pressure (Deville-Bonne & Else, 1991). The stoichiometry of Fru-6P binding to PFK is quite unexpected and corresponds to only two Fru-6P bound per tetramer, i.e., half a Fru-6P per PFK subunit (Figure 1), whereas the presence of four sites in the structure of PFK is clear from X-ray crystallography (Shirakihara & Evans, 1988). This low stoichiometry in Fru-6P binding is not due to partial denaturation of PFK since the same protein samples bind either four ATP or only two Fru-6P.

In the presence of saturating ATP, no or very little enzymatic activity of PFK is observed below 200 μM Fru-6P (Blangy et al., 1968; Le Bras & Garel, 1982). The present results show that, in the absence of ATP, PFK can bind two Fru-6P noncooperatively with a dissociation constant of 32 μM. This difference suggests that the presence of ATP could in-

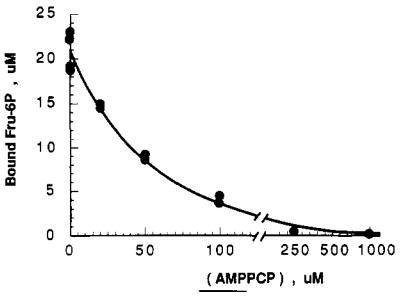

FIGURE 2: Dissociation of Fru-6P from the PFK–Fru-6P complex induced by AMPPCP and measured by equilibrium dialysis: PFK (20 μM in tetramer) was incubated in the presence of 45 μM total Fru-6P (which distributes nearly equally between bound and free Fru-6P) and various amounts of AMPPCP.

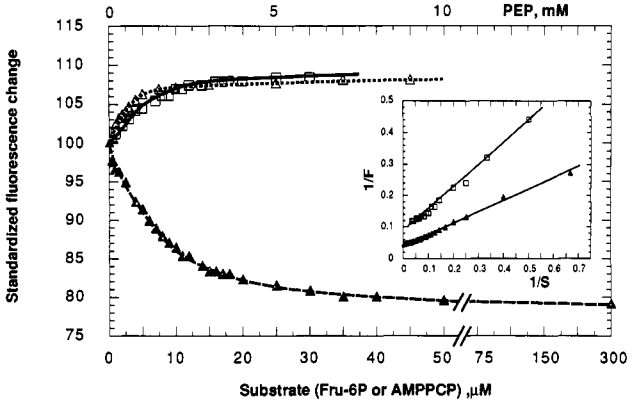

FIGURE 3: Fluorescence change induced by ligand binding to PFK. The standardized fluorescence of PFK (0.7 μM in subunit) has been monitored upon addition of Fru-6P (▲), AMPPCP (□), and PEP (△). The fluorescence emitted by free PFK is given an intensity of 100. The lines are drawn according to the best hyperbolic fit. Inset: Double-reciprocal plot of fluorometric titration by Fru-6P (▲) and AMPPCP (□). The slight deviation from linearity is due to a weak cooperativity (n_H = 1.12).

fluence the binding of Fru-6P. The binding of Fru-6P was studied in the presence of AMPPCP, a nonhydrolyzable analogue of ATP. AMPPCP is indeed a competitive inhibitor of PFK, with an inhibition constant of 60 μM (Blangy, 1971). Figure 2 shows that increasing concentrations of AMPPCP can completely chase the bound Fru-6P from PFK and that an AMPPCP concentration of 50 μM is needed to displace half of the bound Fru-6P. Also, in the presence of 5 mM AMPPCP, no binding of Fru-6P to PFK could be detected up to the highest Fru-6P concentrations compatible with equilibrium dialysis, about 500 μM. This suggests that PFK can bind either Fru-6P or AMPPCP, but not both, under these conditions.

Fluorescence Measurements of the Formation of the Binary Complex between PFK and Fru-6P. The intrinsic fluorescence of PFK is mainly due to its unique tryptophan residue, Trp-311 (Hellinga & Evans, 1985). Although Trp-311 is not located in the active site, its fluorescence is sensitive to the binding of Fru-6P (Berger & Evans, 1991). Figure 3 shows that the fluorescence emitted at 340 nm upon excitation of PFK at 295 nm decreases following a hyperbolic dependence on the concentration of Fru-6P, and is quenched by about 20% at saturation in Fru-6P. The very low extent of cooperativity in Fru-6P binding is shown by the linearity of the double-reciprocal plot (inset of Figure 3) and by the values of n_H lower than 1.2 obtained from fitting the data to the Hill equation. The actual stoichiometry of Fru-6P binding to PFK can be assumed to be the same as that determined by equilibrium dialysis, i.e., half a Fru-6P per PFK subunit. The apparent dissociation constant has a value of 8 μM (mean of five experiments), independent of protein concentration in the range from 0.3 to 2 μM PFK subunit.

Fluorescence Measurements of the Formation of the Binary Complex between PFK and ATP. The fluorescence of Trp-311 is also modified upon binding of the other substrate ATP to PFK. In the presence of a saturating concentration of ATP or its analogue AMPPCP, the fluorescence emitted at 340 nm (upon excitation at 295 nm) increases by 10%, without any significant shift in the emission spectrum. The saturation curve of PFK by AMPPCP (Figure 3) is close to hyperbolic, as shown by the quasi-linearity of its double-reciprocal plot (inset of Figure 3), with an apparent dissociation constant of 5 μM. The saturation of PFK by ATP is also hyperbolic, with an apparent dissociation constant of 3 μM (result not shown).

Fluorescence Measurements of the Binding to PFK of the Allosteric Effectors ADP and PEP. The active sites, which bind ATP and Fru-6P, and the regulatory sites, which bind the allosteric effectors ADP or PEP, are distant in the three-dimensional structure of PFK (Shirakihara & Evans, 1988). Nevertheless, the fluorescence of Trp-311 is also sensitive to the binding of allosteric effectors to PFK: the fluorescence intensity is increased upon binding the inhibitor PEP (Figure 3), whereas it is decreased upon binding the activator ADP, as already observed by Berger and Evans (1991). The saturation curve of PFK by PEP is hyperbolic and corresponds to a dissociation constant of 0.5 mM and a maximum fluorescence increase of 8% (Figure 3). The binding of ADP to PFK causes a maximum fluorescence decrease of 20%, which is an amplitude similar to that due to Fru-6P binding (see above).

Figure 3 and other data show that when PFK is saturated by one of its ligands, the intrinsic fluorescence of the protein has either a high or a low intensity. The fluorescence is increased by 8–10% upon binding of AMPPCP, ATP, or PEP, and decreased by 20% upon binding of Fru-6P or ADP. Inasmuch as fluorescence intensity is an index of protein conformation, only two states seem possible for the binary complexes between PFK and one of its ligands: one conformational state has a high fluorescence (108–110% as compared to free PFK) and the other has a low fluorescence (80% of that of free PFK). The high-fluorescence state, which is favored by the binding of PEP, corresponds probably to the conformation described by Schirmer and Evans (1990) for the binary complex between PFK and PEP. The low-fluorescence state, which is favored by the binding of ADP, could correspond to the conformation described by Shirakihara and Evans (1988) for the ternary complex between PFK, ADP, and Fru-1,6P_2. The states with a high and a low fluorescence could therefore correspond to the T and the R states of the concerted model (Blangy et al., 1968). Its intermediate fluorescence suggests that free PFK corresponds to a mixture of R and T states, with about one-third R and two-third T. This is different from the ratio T_0/R_0 of 4×10^6 between the T and R states of PFK in the absence of ligand derived from steady-state measurements of activity (Blangy et al., 1968), but is in agreement with the ratio T_0/R_0 around 2 obtained by equilibrium dialysis measurements of GDP binding (Blangy, 1971). According to the concerted model, the saturation by a substrate of an allosteric enzyme will be almost hyperbolic when the ratio T_0/R_0 is close to unity (Monod et al., 1965). Therefore, a value of only 2 for the ratio T_0/R_0 can explain the noncooperative binding of Fru-6P or ATP to PFK.

The concerted model also predicts that the cooperativity of Fru-6P binding will increase if PFK is mainly in the T con-

Table I: Values of the Half-Saturating Concentration, $[Fru\text{-}6P]_{1/2}$, and the Cooperativity Coefficient, n_H, for Saturation Curves of PFK by Fru-6P and in the Presence of AMPPCP or PEP[a]

ligand	$[Fru\text{-}6P]_{1/2}$ (mM)	n_H
	0.01	1
0.1 mM AMPPCP	0.21	2.1
0.5 mM AMPPCP	0.97	2.1
1 mM AMPPCP	2.9	2.0
2 mM PEP	0.064	1.9

[a] Data from Figures 3 and 4A,B.

formation initially. It was not possible to measure accurately the entire curve for the binding of Fru-6P by equilibrium dialysis in the presence of AMPPCP, because of the low specific radioactivity of commercially available Fru-6P and the limited affinity of PFK. The cooperative binding of Fru-6P in the presence of AMPPCP could however be quantitatively confirmed by experiments such as that shown in Figure 2 and performed at different Fru-6P concentrations. Fluorescence does not have the same limitations as equilibrium dialysis. The presence of AMPPCP, ATP, or PEP shifts the conformation of PFK toward the high-fluorescence state. The hypothesis made above that a high fluorescence is associated with the T conformation can be further tested by studying the binding of Fru-6P to the binary complexes PFK–AMPPCP and PFK–PEP.

Binding of Fru-6P to PFK in the Presence of AMPPCP or PEP As Measured by Fluorescence Changes. Figure 4A shows the fluorescence changes induced by Fru-6P binding to PFK in the presence of different concentrations of AMPPCP. The presence of AMPPCP modifies the saturation curve of PFK by Fru-6P in three ways: (i) the amplitude of the fluorescence decrease is larger, since it starts from an initial intensity of 110% corresponding to the PFK–AMPPCP complex, instead of the 100% of free PFK, and reaches the same final intensity of about 80% (compare Figures 3 and 4A); (ii) the higher the AMPPCP concentration, the higher the half-saturating Fru-6P concentration (Figure 4A and Table I); (iii) in the presence of AMPPCP, the apparent saturation of PFK by Fru-6P is no longer hyperbolic but becomes sigmoïdal, with a cooperativity coefficient n_H of 2 ± 0.2 (Figure 4A and Table I). The same features are observed for the changes in the fluorescence of PFK upon binding Fru-6P in the presence of PEP (Figure 4B and Table I). This shows that binding of Fru-6P to the high-fluorescence state of PFK does not depend on whether PEP or AMPPCP is present and suggests that PFK is in the same T state when PEP, AMPPCP, or presumably ATP is bound.

It is surprising that the same high-fluorescence T conformation is favored by AMPPCP, which is an analogue of the substrate ATP, and by PEP, which is an inhibitor. This shift toward the T state explains that AMPPCP resembles an allosteric inhibitor (Monod et al., 1965; Blangy et al., 1968); it increases the cooperativity and decreases the apparent affinity of Fru-6P binding to PFK (Figure 4A and Table I). It is remarkable that the cooperative binding of Fru-6P, which is thought to be a prominent feature of the regulatory properties of PFK, results only from an "inhibitor-like" effect of AMPPCP, and presumably of ATP, on Fru-6P.

Binding of AMPPCP and PEP to PFK in the Presence of Fru-6P As Measured by Fluorescence Changes. The "inhibitor-like" effect of AMPPCP on the binding of Fru-6P by PFK can be directly monitored. Indeed, the concerted model predicts that the binding to PFK of an inhibitor which favors the T state should become cooperative in the presence of Fru-6P, which favors the R state (Monod et al., 1965).

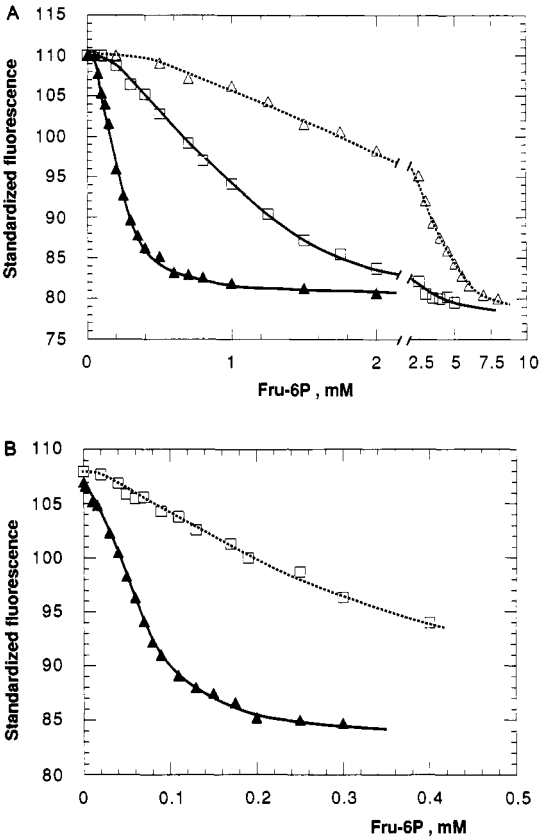

FIGURE 4: (A) Fluorescence change of PFK upon binding Fru-6P in the presence of AMPPCP. PFK (1.2 μM in subunit) is in the presence of 0.1 (▲), 0.5 (□), or 1 mM AMPPCP (▲). The fluorescence of the initial free PFK has been taken as 100. (B) Fluorescence change of PFK upon binding Fru-6P in the presence of PEP. PFK (1 μM in subunit) is in the presence of 2 (▲) or 8 mM (□) PEP. 100 is the fluorescence of the free protein.

Figure 5 shows that this is the case: a cooperative saturation curve from a low-fluorescence state (80%) to a high-fluorescence state (105–110%) is observed with eigher AMPPCP (Figure 5A) or PEP (Figure 5B). Also, the half-saturating concentration of AMPPCP or PEP increases with the concentration of Fru-6P present (Figure 5). For instance, that of AMPPCP increases from 0.037 to 0.12 mM when Fru-6P increases from 0.1 to 0.3 mM (Figure 5A). The cooperativity coefficient n_H, however, seems to remain around the value of 2, independently of Fru-6P concentration: for the binding of AMPPCP, n_H is 1.75 and 2.2 at 0.1 and 0.3 mM Fru-6P, respectively (inset of Figure 5A), and for the binding of PEP, n_H is 1.9 at 0.1 mM Fru-6P (inset of Figure 5B).

X-ray crystallography has shown that the T state of PFK obtained upon binding PEP is probably unable (or almost) to bind Fru-6P (Schirmer & Evans, 1990), so that the inhibition of Fru-6P binding by PEP is explained by the conformational shift from the R to the T state. The binary complex between AMPPCP and PFK resembles that between PEP and PFK and thus appears as a T state both structurally, by its high fluorescence (Figure 3), and functionally, by its cooperative binding of Fru-6P (Figure 4A). Therefore, AMPPCP and presumably ATP seem to shift the conformation of PFK toward the same T state as PEP. The lower affinity of PFK for Fru-6P in the presence of AMPPCP is in agreement with the chase of bound Fru-6P by AMPPCP observed in equilibrium dialysis (Figure 2).

Discussion

Equilibrium dialysis and fluorescence lead to slightly dif-

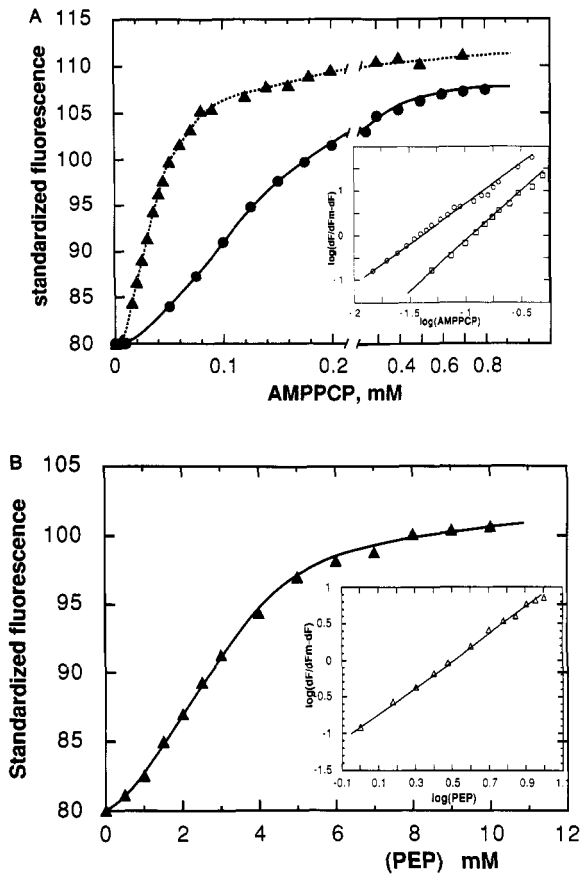

FIGURE 5: (A) Fluorescence change of PFK upon binding AMPPCP in the presence of Fru-6P. PFK (0.7 μM in subunit) is incubated with 0.1 (▲) or 0.3 mM (●) Fru-6P. Fluorescence is expressed relatively to the free protein, taken as 100. Inset: Hill plot of the same data: Fru-6P, 0.1 (○) or 0.3 mM (□). (B) Fluorescence change of PFK upon binding PEP in the presence of Fru-6P. PFK fluorescence (0.7 μM in subunit) in the presence of 0.1 mM Fru-6P, expressed relatively to the fluorescence of the free protein, is monitored upon PEP addition. Inset: Hill plot of the same data.

ferent mean values for the dissociation constant of the same ligand. It seems unlikely that this difference is due to the 10–50 times larger PFK concentration in equilibrium dialysis since *E. coli* PFK remains tetrameric up to 150 μM in subunit (Blangy, 1968; P. R. Evans, personal communication). A statistical analysis of all the equilibrium dialysis data for Fru-6P binding (Johnson & Frazier, 1985) shows that the dissociation constant is not measured accurately and lies between 10 and 87 μM with a mean value of 32 μM. The same analysis for fluorescence measurements of Fru-6P binding gives a dissociation constant between 6 and 11 μM with a mean value of 8 μM. A similar dispersion is observed for ATP and AMPPCP. The 3–4-fold difference in the mean values obtained by the two methods does not seem statistically significant. A value of 20 μM has been recently reported for Fru-6P dissociation by Berger and Evans (1991) and lies between the 8 and 32 μM found here. In addition, two sources of error could explained the lower values determined by fluorescence: (i) all ligands of PFK are phosphate derivatives, which quench the protein fluorescence. Our correction for nonspecific changes (see Materials and Methods) probably introduces some error in the determination of the fluorescence of PFK at saturation in Fru-6P; (ii) as pointed out by Ward (1985), changes in fluorescence intensity may not be proportional to the degree of saturation. For instance, the binding of the first Fru-6P could induce a larger fluorescence change than that of the second Fru-6P, thus leading to an underestimation of the actual dissociation constant.

The intrinsic fluorescence of the binary complexes between *E. coli* PFK and one of its ligands suggests that the protein exists under two conformations only, with respectively a high and a low fluorescence. The binding properties of these two conformations, determined by equilibrium dialysis and/or fluorescence changes, show that the high-fluorescence state binds the inhibitor PEP, the substrate ATP, and its analogue AMPPCP while the low-fluorescence state binds the activator ADP and the substrate Fru-6P. This behavior is qualitatively in agreement with a unique transition between the two states R and T postulated by the concerted model (Monod et al., 1965), the T state having a high fluorescence and the R state a low fluorescence.

Free PFK is close to an equimolar mixture of R and T states (the ratio T_0/R_0 is around 2), so that no cooperativity is detected in the binding of any ligand, whether of Fru-6P to the R state (Figures 1 and 3) or of ATP (or AMPPCP) to the T state (Figure 3). Cooperativity is, however, detected when binding of a ligand involves the complete shift from one conformation to the other (Figures 4 and 5), which suggests that some interactions between sites exist within the PFK molecule. Therefore, the concerted model of allosteric transition (Monod et al., 1965) seems to apply, at least qualitatively, to the structural and functional properties of PFK, as studied here by fluorescence and ligand binding.

Several results are, however, not in agreement with earlier conclusions about PFK. First, the cooperativity observed for the binding of Fru-6P is limited: the Hill coefficient n_H for binding is only around 2 (Figure 4), which suggests that only two Fru-6P sites are in the binding interaction. This Hill coefficent n_H of 2 is much lower than that obtained from steady-state measurements of activity, where values of n_H of 3.8–4 are found (Blangy et al., 1968). This difference in n_H values indicates that the remarkable overall cooperativity of Fru-6P saturation is only partially due to the intrinsic cooperativity of Fru-6P binding and contains an important kinetic contribution from the subsequent steps of the enzymatic reaction. Previous results have already shown that PFK behaves as a mixed allosteric enzyme with both binding and kinetic cooperativity (Deville-Bonne et al., 1991a). A difference in cooperativity could also exist between binding and steady-state activity measurements of the saturation of PFK by ATP. If the binding of ATP is the same as that of AMPPCP, it is cooperative in the presence of Fru-6P (Figure 5A), whereas activity data show a hyperbolic saturation (Blangy et al., 1968). Therefore, no clear relationship exists between the initial rate of PFK-catalyzed reaction and the extent of binding of ATP and/or Fru-6P. Dalziel (1968) has emphasized the possible complexity of such relationships.

The two substrates of PFK are binding antagonists since ATP binds to the T state and Fru-6P to the R state. This antagonism is partially detected by activity measurements since the apparent Michaelis constant for one substrate increases with the concentration of the other substrate (Kundrot & Evans, 1991; Deville-Bonne et al., 1991b). The conformation of PFK which is catalytically active is not known exactly, but it is probable that phosphate transfer takes place in the R or an "R-like" state (Shirakihara & Evans, 1988). The inactive T conformation taken by the binary ATP–PFK complex could be needed to avoid the undesired transfer of phosphate on a water molecule, i.e., to prevent any ATPase activity.

Four active sites are visible in the three-dimensional structure of tetrameric PFK (Shirakihara & Evans, 1988), and indeed the binding of four ATP can be measured (Blangy,

1971; this work). However, the same PFK tetramer binds only two Fru-6P (Figure 1), in a sort of half-of-the-sites reactivity. This stoichiometry of half a ligand per subunit resembles that of some aminoacyl-tRNA synthetases, which bind one amino acid and two ATP per dimer (Blanquet et al., 1972; Fersht, 1985). The binding of Fru-6P to only two out of the four sites results in a partial asymmetry of the protein. The PFKs from *E. coli* (Shirakihara & Evans, 1988) and *B. stearothermophilus* (Evans et al., 1981) have the structure of a dimer of dimers, for which some asymmetry has already been reported: in the *B. stearothermophilus* enzyme, the active site is only half-occupied by the product ADP, and in the *E. coli* enzyme, the active sites in two subunits are in one "open" conformation and the sites in the two other subunits are in a different "closed" conformation. In both *E. coli* and *B. stearothermophilus* PFKs, two Fru-6P binding sites are spatially close to one another and related by a 2-fold symmetry axis (Evans et al., 1981; Shirakihara & Evans, 1988). The binding of the first Fru-6P to one site could distort the neighboring site and impair its ability to bind Fru-6P, thus leading to the observed stoichiometry of one Fru-6P bound per PFK dimer. Independently of its origin, the half-of-the-sites reactivity in Fru-6P binding explains that the corresponding cooperativity coefficient does not exceed the value of $n_H = 2$ (Table I). It is not known if all four active sites function at the same time and are actually involved in the values of n_H around 4 measured by steady-state activity, but this half-of-the-sites reactivity could be relevant to the catalytic mechanism of PFK. Indeed, the correlation between n_H and the number of sites is not straight, and values of n_H as large as 5.5–6 have been measured for only four sites (Deville-Bonne et al., 1991a).

In conclusion, PFK represents a paradoxical enzyme which seems to obey a concerted allosteric mechanism in two different ways. First, the equilibrium data about ligand binding and conformation changes are consistent with a transition between two states, a low-fluorescence R state which binds the substrate Fru-6P and the activator ADP and a high-fluorescence T state which binds the other substrate ATP and the inhibitor PEP, with a ratio of T_0/R_0 around 2 for free PFK. Second, the steady-state measurements of activity are also consistent with a concerted transition between two states, a R state which has a high apparent affinity for the substrate Fru-6P and would bind the activator ADP and T state which would bind the inhibitor PEP; the ratio between two states would be T_0/R_0 around 4×10^6 for free PFK, and the R and T states would have the same apparent affinity for the other substrate ATP (Blangy et al., 1968). This major discrepancy between thermodynamic and kinetic data suggests that the PFK-catalyzed reaction does not operate at quasi-equilibrium and that both binding and catalytic steps are involved in allosteric control of activity. Despite the quality of structural data (Shirakihara & Evans, 1988; Rypniewski & Evans, 1989; Schirmer & Evans, 1990), the remarkable analysis of the steady-state behavior using the concerted model (Blangy et al., 1968), and the surprising properties of some mutated PFKs (Lau & Fersht, 1987; Serre et al., 1990; Berger & Evans, 1990), basic enzymatic information about the rate-limiting step, the existence of conformational changes, and the kinetic mechanism is still missing to understand the delicate mechanism by which PFK regulates its activity.

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