Steady-State Fluorescence of *Escherichia coli* Phosphofructokinase Reveals a Regulatory Role for ATP†

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ABSTRACT: We have investigated the effects of ligands and effectors on the intrinsic fluorescence of *Escherichia coli* phosphofructokinase (PFK). We have found that the substrate fructose 6-phosphate (Fru6P) or the allosteric activator ADP can quench the fluorescence up to 35%. The response is hyperbolic with $K_{s[Fru6P]}$ of 20 μM and $K_{s[ADP]}$ of 13 μM. The allosteric inhibitor phosphoenolpyruvate (PEP) converts the hyperbolic response with respect to Fru6P to a sigmoidal response. AMP-PNP, a nonhydrolyzable analogue of ATP, also inhibits the Fru6P fluorescence response. PFK mutant KA213, which is insensitive to effectors, has a decreased fluorescence response with respect to ADP, and PEP does not convert the Fru6P response to sigmoidicity. However, its fluorescence response with respect to Fru6P is decreased by ATP or AMP-PNP. Taken together, these results suggest that, in the absence of effectors or ligands, *E. coli* PFK exists in a state with high affinity for Fru6P ("R" state). This state can be altered to a low affinity ("T" state) by PEP binding to the allosteric site or by ATP binding to the enzyme.

Phosphofructokinase (PFK) catalyzes the phosphorylation of fructose 6-phosphate (Fru6P) to fructose 1,6-bisphosphate (Fru1,6BP). This reaction represents the key commitment step in glycolysis and is tightly regulated in a wide variety of organisms (Uyeda, 1979). The enzyme from *Escherichia coli* (PFK-1; EC 2.7.1.11) is a tetramer and displays highly cooperative kinetics with respect to Fru6P, hyperbolic kinetics with respect to ATP, allosteric inhibition by phosphoenolpyruvate (PEP), and activation by ADP or GDP (Blangy et al., 1968). The kinetic and structural properties of this enzyme are consistent with a simple two-state model where the principal difference between the two states is their affinity for Fru6P (Blangy et al., 1968, Schirmer & Evans, 1990). The low-affinity (T) state is stabilized by the allosteric inhibitor PEP, while the high-affinity (R) state is stabilized by the allosteric activator ADP. However, this is a model for the binding of ligands rather than of the kinetics and needs to be confirmed by direct measurement of ligand binding. One simple method of measuring binding is from the change of intrinsic fluorescence with ligand concentration [see e.g., Schmid (1989)]. *E. coli* PFK contains a single tryptophan Trp311 remote from the active site but in a subunit interface. The fluorescence of this tryptophan has been shown to be sensitive to denaturation (Le Bras & Garel, 1989).

We report here the steady-state fluorescence properties of native PFK in the presence and absence of ligands. Our results demonstrate that the intrinsic fluorescence of *E. coli* PFK is sensitive to the conformational changes associated with substrate and effector binding. They also reveal a previously unknown role for ATP in the regulation of this enzyme.

Materials and Methods

Enzymes and Reagents. Cultures of DF1020 cells (which are deleted for both PFK genes) containing either the vector pHL1 (Lau et al., 1987), which contains the wild-type *E. coli* PFK gene, or pKA213 (Lau & Fersht, 1987), which contains the Lys → Ala213 mutant, were grown to confluence in the presence of 70 μg/mL IPTG. Cells were pelleted, resuspended in 1/40th of their original volume of TE (50 mM, Tris, pH 8.0, 1 mM EDTA, 2 mM DTT) and stored at −20 °C. Frozen cells were thawed and sonicated, and the supernatant was clarified by centrifugation. PFK was purified from the supernatant by using a Cibacron blue-A column (Berger & Evans, 1990; Hellinga & Evans, 1987) and stored as a 55% ammonium sulfate precipitate (Kotlarz & Buc, 1982). As *E. coli* PFK is sensitive to air oxidation (Banas et al., 1988), all PFK solutions were stored in 10 mM DTT under argon.

Aliquots of PFK were desalted on a Sephacryl S-200 column (Pharmacia). Protein concentration was measured by absorbance [specific absorbance ϵ_{278} = 0.6 cm^2 mg^{-1} (Kotlarz & Buc, 1977)]. All reagents used in this study were from Boehringer Mannheim. Measurements containing ADP or ATP also included 10 mM $MgCl_2$ and 10 mM NH_4Cl.

Fluorescence Measurements. Fluorescence measurements were performed at 26 °C with a Perkin-Elmer LS-5B luminescence spectrometer. Excitation and emission slits were set to 10 nm. The excitation wavelength was set to 295 nm to limit fluorescence to tryptophans only. The protein concentration was 15–30 μg/mL (0.5–1.0 μM), within the linear portion of the fluorescence vs concentration curve. Fluorescence spectra from 320–360 nm were collected by using data capture software written by J. Foote (MRC LMB) and run on an Apple Macintosh computer. Background readings were subtracted, and the wavelength and value of the fluorescence maxima were recorded. Experiments were performed by adding small increments of substrate or effector to the enzyme solution. Corrections were made to compensate for volume changes and enzyme dilution. Maximum fluorescence change and K_s values were obtained by fitting data to either the hyperbolic (Michaelis–Menten) equation [$F = F_{max}S/(S + K_s)$] or the Hill equation [$F = F_{max}S^n/(S^n + S_{1/2}^n)$] with a program written by S.A.B. using the AMOEBA nonlinear least-squares curve-fitting algorithm from Press (1988). Experimental errors on the fluorescence measurements were estimated to be ±10%. Errors, where reported, were derived from the curve-fitting

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Table I: Kinetic Parameters of Wild-Type and KA213 PFKs[a]

	k_{cat} (s^{-1})	$K_{m[Fru6P]}$ (μM)	$K_{m[ATP]}$ (μM)	$S_{1/2}$ (μM)	n_H	$K_{R[ADP]}$ (μM)	$K_{T[PEP]}$ (mM)
wt	130	30	60	540	4.0	15	0.7
KA213	140	22	40	180	3.3	200	27

[a] k_{cat} values were measured at saturating concentrations of both substrates. The $K_{m[Fru6P]}$ value for the wt enzyme was measured in the presence of 1 mM GDP (GDP and ADP both activate the enzyme: GDP is used for kinetic measurements as it is a poor substrate for the reverse reaction, but it is less suitable for fluorescence measurements). The $K_{m[Fru6P]}$ value for KA213 was estimated by fitting the kinetic data to the Monod–Wyman–Changeux model (Blangy et al., 1968). $K_{m[ATP]}$ values were obtained at saturating values of Fru6P. $S_{1/2}$ is the Fru6P concentration at half-maximal velocity; n_H is the Hill constant determined from fitting kinetic data to $V = V_{max}S^n/(S_{1/2}^n + S^n)$. $K_{r[ADP]}$ and $K_{T[PEP]}$ values are from Blangy et al. (1968) and Lau (1989).

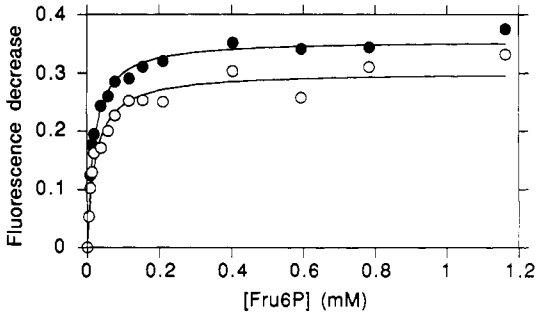

FIGURE 1: Fluorescence decrease versus Fru6P. The fractional fluorescence decrease is plotted as a function of increasing concentrations of Fru6P. (●) Wild-type enzyme. (○) KA213. The curves are fitted to the hyperbolic equation.

algorithm as described (Press, 1988).

Results

Fru6P. As previously reported (Tescher & Garel, 1989), PFK exhibits a fluoresence excitation spectrum with a maximum at 280 nm. The fluorescence signal is reduced to 36% at 295 nm. This signal at 295 nm is presumably due to the single Trp at residue 311. Excitation at 295 nm produces a fluorescence emission with a maximum at 337 ± 2 nm. Upon addition of saturating concentrations of Fru6P (2 mM), the fluorescence signal of both wild-type and mutant PFKs is quenched by 35% of its initial value. The concentration dependence of the quenching is hyperbolic (Hill coefficient between 0.9 and 1.1) with a $K_{s[Fru6P]}$ of 20 ± 2 μM for both wild-type and mutant (Figure 1).

A hyperbolic response is indicative of saturable ligand binding to a single state (or several states with equal affinity). Furthermore, the dissociation constant $K_{s[Fru6P]}$ value measured here is slightly lower than the value of 30 μM obtained for K_m from activity measurements of the activated R-state enzyme (Table I). This is consistent with the fact that $K_m = K_s + k_3/k_1$. This low value for the K_s suggests that in the absence of ligands, PFK exists primarily in the R state. This is in contrast to the result obtained from activity measurements where, in the presence of saturating concentrations of ATP, the kinetics with respect to Fru6P are highly sigmoidal with an $S_{1/2}$ of 540 μM and a Hill coefficient of approximately 4 (Table I). These sigmoidal kinetics have been interpreted to suggest that the unliganded enzyme is primarily in an inactive T state characterized by an estimated $K_{T[Fru6P]}$ of 25 mM (Blangy et al., 1968). With increasing concentrations of Fru6P, the equilibrium would shift to the more active R state, leading to increased binding and catalytic activity.

ADP and PEP. To explore further the nature of the unliganded state, we investigated the effect of the allosteric effectors ADP and PEP on the intrinsic fluorescence. (GDP was not used for these measurements since it absorbs significantly at 295 nm.) ADP causes a similar quenching of wild-type fluorescence as Fru6P (Figure 2). The response is hyperbolic with a maximal fluorescence decrease of 23%

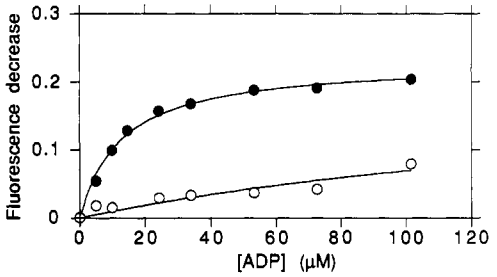

FIGURE 2: Fluorescence decrease versus ADP. The fluorescence decrease is plotted as a function of increasing concentrations of ADP. (●) Wild-type enzyme. (○) KA213. The curves are fitted to the hyperbolic equation.

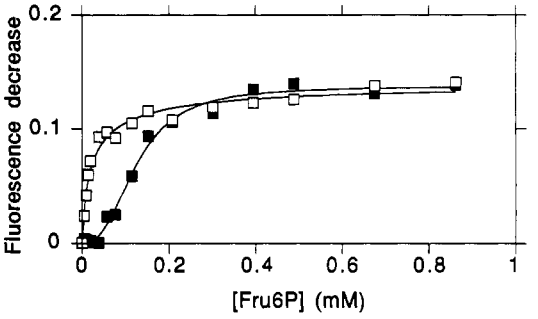

FIGURE 3: Fluorescence response modified by PEP. The fluorescence decrease versus Fru6P is shown in the presence of 2 mM PEP. (■) Wild-type. (□) KA213. The curves are fitted to the Hill equation.

and a $K_{s[ADP]}$ of 13 ± 3 μM. In contrast, the mutant KA213 is insensitive to quenching by ADP. The $K_{s[ADP]}$ for this mutant is probably greater than 200 μM. This agrees with the insensitivity of this mutant to activation by dinucleotides as measured by catalytic activity (Lau & Fersht, 1987; Table I).

PEP induces only minor changes in the fluorescence of PFK with a maximum decrease of 5% (not shown). However, it causes the fluorescence change of the wild-type enzyme with respect to Fru6P to become sigmoidal. As shown in Figure 3, at 2 mM PEP, the fluorescence response of the wild-type enzyme is sigmoidal with a Hill coefficient of 2.6 ± 0.3. In contrast, the response of the mutant KA213 is unchanged and remains hyperbolic (Hill constant of 0.8 ± 0.1).

The observation that Fru6P causes a hyperbolic change in the fluorescence with a low K_s, coupled with the fact that PEP converts this fluorescence response to sigmoidicity, suggests that in the absence of ligands PFK exists primarily in a state with high affinity for Fru6P. This contrasts with previous interpretations based on activity measurements which suggest that, in the absence of Fru6P, PFK is primarily in the low-affinity T state. One difference between the activity and fluorescence measurements, however, is that activity measurements are performed in the presence of saturating ATP concentrations. We therefore investigated the effect of ATP and its nonhydrolyzable analogue AMP-PNP on the steady-state fluorescence.

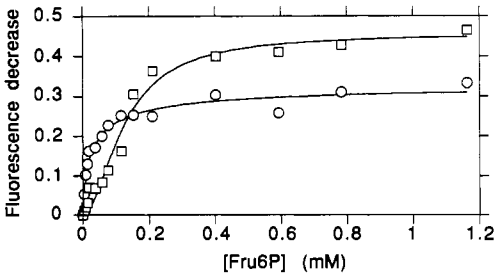

FIGURE 4: Effect of ATP on the fluorescence response of KA213. Fluorescence decrease with respect to Fru6P was measured in the presence or absence of 100 μM ATP. (○) No ATP. (□) With ATP. The curves are fitted to the Hill equation.

ATP and AMP-PNP. ATP causes a slight increase in fluorescence (5%) at low concentrations, followed by a gradual decrease. AMP-PNP shows a similar response. The effect of ATP on the binding of Fru6P by the wild-type enzyme cannot be measured directly because the reaction would produce the activator ADP. It can, however, be used with the mutant KA213, which is insensitive to ADP activation. As shown in Figure 4, the fluorescence change of KA213 with respect to Fru6P in the presence of ATP is inhibited at low concentrations, and the dependence is perhaps slightly sigmoidal (Hill coefficient of 1.8 ± 0.3). With increasing concentrations of Fru6P, the fluorescence decreases to the same extent as in the absence of ATP. At least two interpretations are suggested by this result. First, ATP may simply be a substrate inhibitor with respect to Fru6P. The gradual decrease in fluorescence with increasing concentrations of Fru6P would be a result of the depletion of ATP as the reaction proceeds. Alternatively, ATP may be acting to shift the equilibrium toward the T state. This would result in sigmoidal kinetics with respect to Fru6P in a fashion similar to that of PEP. To test this, we investigated the effect of the nonhydrolyzable analogue AMP-PNP on the fluorescence response with respect to Fru6P. As shown in Figure 5, both wild-type and KA213 are inhibited by AMP-PNP at low Fru6P concentrations. This inhibition is overcome at higher Fru6P concentrations, but the response does not appear to be sigmoidal.

Discussion

In this study, we have shown that binding of Fru6P to *E. coli* PFK causes a hyperbolic decrease in the intrinsic fluorescence. This response becomes sigmoidal upon addition of PEP or ATP. AMP-PNP also inhibits the fluorescence at low Fru6P concentrations although the binding does not seem to be sigmoidal: this may be due to differences between AMP-PNP and ATP. ADP also causes a decrease in the fluorescence, although not to the same extent as with Fru6P. Furthermore, the intrinsic fluorescence of KA213, an effector-site mutant that is insensitive to activation by ADP or inhibition by PEP, is not affected by the addition of these effector molecules.

Although the fluorescence change allows us to measure binding of ligands, it is not clear what causes the change. Indeed, the changes due to ADP binding may be different from those due to Fru6P. The single Trp311 lies in the subunit interface within the dimer that does not change during the allosteric transition (Schirmer & Evans, 1990), so the shift from T to R state would not be expected to cause a major change in fluorescence. Binding of the effectors ADP or PEP protects against proteolysis (Le Bras & Garel, 1985), presumably by ordering the C-terminal region including Trp311, but PEP causes only a small change in fluorescence, unlike

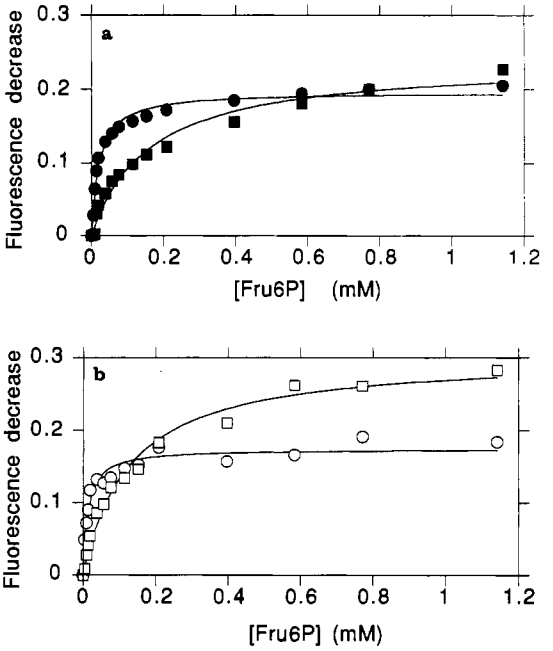

FIGURE 5: Effect of AMP-PNP on fluorescence response. (a) Wild-type enzyme. (b) KA213. (○, ●) No AMP-PNP. (□, ■) +10 μM AMP-PNP. The curves are fitted with the hyperbolic equation.

ADP, so ordering of the C-terminal region also cannot be solely responsible for the fluorescence change.

Kinetic measurements have suggested that, in the absence of Fru6P, PFK exists primarily in the inactive T state, while our fluorescence measurements suggest that the unliganded form of PFK is primarily in the R state, though the measurements also show the antagonism between Fru6P and ATP as inhibition by ATP at low Fru6P concentrations [see Figure 4 of Kundrot and Evans (1991)]. The kinetic and fluorescence data can be at least partly reconciled if one proposes that ATP acts as an "allosteric" inhibitor. Kinetic measurements with respect to Fru6P are usually measured in the presence of saturating amounts of ATP. If ATP stabilizes the T state, then the kinetics with respect to Fru6P, as measured by activity, would be sigmoidal as is observed. The data obtained in this study provide some support for such a role of ATP. Both ATP and AMP-PNP increase the fluorescence slightly, and both cause inhibition of the fluorescence change at low concentrations of Fru6P. However, the inhibited response to Fru6P in the presence of AMPPNP is not sigmoidal, and that with ATP is only slightly sigmoidal, so this binding model cannot explain fully the highly sigmoidal kinetics. A satisfactory model for the kinetics may require consideration of changes in k_{cat} as well as K_s as a function of substrate saturation.

These results also explain the previous observation that crystals of unliganded PFK seem to be in the R state configuration (Rypniewski & Evans, 1989). They are also consistent with the observation that ATP converts the concentration dependence with respect to Fru6P for protection of PFK against proteolysis from a hyperbolic to a sigmoidal response (Le-Bras & Garel, 1985). As well, despite the high homology between PFK from *Bacillus stearothermophilus* and *E. coli*, especially in the active site, the *B. stearothermophilus* enzyme exhibits sigmoidal kinetics only in the presence of PEP (Valdez et al., 1989). The differences between the two enzymes may thus reflect slightly different regulatory roles for ATP.

An inhibitory role for ATP is consistent with the regulatory requirements of PFK. Low energy charge requires high activity of this enzyme. Therefore, ADP activates the enzyme.

High energy charge requires low activity thus the inhibition by PEP and ATP. Indeed, ATP is a common inhibitor of PFKs from other organisms as well (Uyeda, 1979).

It is not clear from the structure of the active site how ATP is acting. ATP interacts with Arg72, which we have shown is an important mediator of cooperative signals between subunits (Berger & Evans, 1990). In the T state of the *B. stearothermophilus* enzyme, this residue forms a stabilizing salt bridge with Glu241 from the adjacent subunit. It is not clear how ATP could either stabilize this interaction or bind without interfering with it. Model building of the R-state configuration of ATP in the T-state structure reveals no obvious interactions that might account for the ability of ATP to stabilize the T state. It is formally possible that ATP may be acting through a site distinct from the active site. Perhaps a structural determination in the presence of only ATP might be helpful in explaining this.

Registry No. Fru6P, 643-13-0; PEP, 138-08-9; 5′-ADP, 58-64-0; 5′-ATP, 56-65-5; PFK, 9001-80-3.

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Rational Design of Quinazoline-Based Irreversible Inhibitors of Human Erythrocyte Purine Nucleoside Phosphorylase†

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ABSTRACT: Described herein is the rational design of irreversible inhibitors of human erythrocyte purine nucleoside phosphorylase (PNPase). Inhibitor design started with the observation that the amino group of 8-aminoquinazolin-4(3*H*)-one interacts with enzyme-bound phosphate. This observation correctly predicted that the 5,8-dione (quinone) and 5,8-dihydroxy (hydroquinone) derivatives of quinazolin-4(3*H*)-ones would enter the active site. The amine–phosphate interaction also served to confirm that a quinazolin-4(3*H*)-one binds in the PNPase active sites like a purine substrate. From models of the PNPase active site it was possible to design quinazoline-based quinones that undergo a reductive-addition reaction with an active-site glutamate residue. The best inhibitor studied, 2-(chloromethyl)quinazoline-4,5,8(3*H*)-trione, rapidly inactivates PNPase by a first-order process with an inhibitor to enzyme stoichiometry of 150. The active-site hydroquinone adduct of this inhibitor eliminates a leaving group to afford a quinone methide species positioned to alkylate another active-site glutamate residue. Thus, this inhibitor is designed to cross-link the PNPase active site by reductive addition followed by the generation of an alkylating quinone methide species.

Purine nucleoside phosphorylase (PNPase) catalyzes the reversible phosphorylase of inosine and guanosine to the respective bases and ribose 1-phosphate. The degradative nature of this enzyme initially suggested that PNPase inhibitors would have only limited chemotherapeutic value (Parks & Agarwal, 1972). In the past ten years, however, it became apparent that PNPase inhibitors could be used in the prevention of foreign tissue rejection, in the treatment of gout and malaria, and for the potentiation of antineoplastic nucleosides (Parks et al., 1981; Kazmers et al., 1981; Daddona et al., 1986; Shewach et al., 1986). Thus, there has been a great deal of effort devoted to the design of PNPase inhibitors, most of which are purine, purine nucleoside, or purine nucleotide analogues

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