Ligand-Induced Conformational Transitions in *Escherichia coli* Phosphofructokinase 2: Evidence for an Allosteric Site for $MgATP^{2-}$ †

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ABSTRACT: The binding of ligands to phosphofructokinase 2 (Pfk-2) from *Escherichia coli* induces changes in the fluorescence emission properties of its single tryptophan residue, Trp88, suggesting that upon binding the protein undergoes a conformational change. This fluorescence probe was used to determine the presence of an allosteric site for $MgATP^{2-}$ in the enzyme. Fructose 6-phosphate (fructose-6-P), the first substrate that binds to the enzyme with an ordered bi-bi mechanism, increases the fluorescence up to 30%. The saturation curve for this compound is hyperbolic with a K_d of 6 μM. The titration of Pfk-2 with $MgATP^{2-}$ causes a quenching of fluorescence of about 30% of its initial value, with a blue shift of 7 nm in the emission maximum. The response is cooperative with a K_d of 80 μM and a Hill coefficient of 2. The interaction of $MgATP^{2-}$ cannot take place at the active site in the absence of fructose-6-P, due to the ordered kinetic mechanism. Addition of compounds that bind to the catalytic site of Pfk-2, such as ATP^{4-} or Mg−AMP-PNP, did not produce significant changes in the fluorescence spectrum of Trp88. However, in the absence of Mg^{2+}, the addition of ATP^{4-} to the enzyme−fructose-6-P complex shows a hyperbolic increase of fluorescence of 8%. Acrylamide steady-state quenching experiments for different enzyme−ligand complexes of Pfk-2, indicate that the tryptophan in the enzyme−$MgATP^{2-}$ complex is exposed to a much smaller extent to the solvent than in the free enzyme or in the enzyme−fructose-6-P complex. The effect of the binding of fructose-6-P or $MgATP^{2-}$ on the polarization fluorescence of the emission of Trp88 in Pfk-2 indicates changes in the local mobility of the Trp88 in both enzyme complexes. The average lifetime for Trp88 in Pfk-2 was found to be unusually large, approximately 7.7 ns, and did not vary significantly with the ligation state of the enzyme, which demonstrates that the quenching or enhancement of fluorescence induced by the ligands is mainly due to the complex formation with Pfk-2. These results demonstrate the presence of an allosteric site for $MgATP^{2-}$ in Pfk-2 from *E. coli*, responsible for the inhibition of the enzyme activity by this ligand.

Phosphofructokinase (PFK)[1] catalyzes the phosphorylation of fructose-6-phosphate (fructose-6-P) to fructose-1,6-bisphosphate (fructose-1,6-bisP). This reaction represents the key commitment step in the glycolysis pathway and is highly regulated in a wide variety of organisms. There are two phosphofructokinases in *Escherichia coli.* Pfk-1, the main isozyme, is a tetramer and displays highly cooperative kinetics with respect to fructose-6-P and hyperbolic kinetics with respect to ATP. Its activity is increased by ADP or GDP and is inhibited by phosphoenolpyruvate (*1*, *2*). Pfk-2, the minor isozyme in the wild-type strain, presents hyperbolic kinetics with fructose-6-P and inhibition by $MgATP^{2-}$ when the assay is performed at low fructose-6-P concentrations (*3*). The primary structure of Pfk-2, as predicted from the nucleotide sequence, shows no significant relationship to the Pfk-1 family but appears to be related to another group of kinases, which includes fructose-1-P kinase (*4*). Also, Pfk-1 and Pfk-2 differ as to the order in which the substrates are bound to and the products are released from the enzyme (*5*). Kinetic studies related to the inhibitory effect of $MgATP^{2-}$ on Pfk-2 suggest the presence of an allosteric site for this compound responsible for the inhibition of the enzyme activity (*3*), and it also has been linked to a dimer−tetramer association process (*6*). A regulatory role for $MgATP^{2-}$ also has been proposed for *E. coli* Pfk-1 (*7*). Through the steady-state quenching of Pfk-1 intrinsic tryptophan fluorescence it was demonstrated that fructose-6-P, in the absence of $MgATP^{2-}$, binds to the enzyme with much greater affinity than at saturating $MgATP^{2-}$ concentrations. This mutually antagonistic relationship between the two substrates, $MgATP^{2-}$ and fructose-6-P, has been studied in detail (*8*−*12*).

Since Pfk-2 has an ordered bi-bi mechanism with fructose-6-P being the first substrate to bind and fructose-1,6-bisP the last product to be released, one would not predict binding of $MgATP^{2-}$ in the absence of fructose-6-P. Since the interaction of $MgATP^{2-}$ at an allosteric site in Pfk-2 site has been proposed from kinetics studies (*3*), we decided to determine the presence of this site by measurements of ligand binding, monitoring the changes of intrinsic fluorescence properties of the protein (*13*). *E. coli* Pfk-2 contains only

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[1] Abbreviations: PFK, generic phosphofructokinase; Pfk-1, phosphofructokinase 1; Pfk-2, phosphofructokinase 2; fructose-6-P, fructose 6-phosphate; fructose-1,6-bisP, fructose 1,6-bisphosphate; P-enolpyruvate, phosphoenolpyruvate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol.

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one tryptophan residue (Trp 88), which makes this enzyme suitable for this kind of study.

Here we report the intrinsic steady-state fluorescence properties of Pfk-2 in the presence and absence of ligands. Our results show that the tryptophan emission of *E. coli* Pfk-2 is very sensitive to the conformational changes associated with substrate or effector binding and that the binding of the inhibitor $MgATP^{2-}$ occurs at an allosteric site.

MATERIALS AND METHODS

Phosphofructokinase Purification. The enzyme was purified from the *E. coli* strain DF 903 as starting material (*1*, *14*).

Enzyme Activity Measurements. PFK activity was carried out spectrophotometrically by coupling the fructose-1,6-bisP formation to the oxidation of NADH with the use of aldolase, triosephosphate isomerase, and α-glycerophosphate dehydrogenase (*1*).

Reagent Concentrations. Fructose-1,6-bisP concentration was estimated spectrophotometrically by the aldolase coupling assay. The concentration of NADH was calculated with a molar extinction coefficient of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$. ATP concentrations were determined spectrophotometrically by measuring the fructose-1,6-bisP formed in the presence of PFK, fructose-6-P, aldolase, triosephosphate isomerase, α-glycerophosphate dehydrogenase, and NADH. Protein concentration was determined as described by Bradford (*15*), with bovine serum albumin as standard.

Fluorescence Measurements. Fluorescence and polarization measurements were performed with a Perkin-Elmer LS 50 fluorometer. Polarization determinations were made using an L format. The excitation wavelength was set to 295 to limit fluorescence to tryptophans only. Emission spectra from 300 to 500 nm were collected and the excitation and emission slits were set to 5 nm. Backgrounds readings were subtracted, and the wavelength and fluorescence maximum values were recorded. All the spectroscopic measurements were collected at room temperature. Titration experiments were performed by adding small aliquots of stock solutions of substrate or effector to the enzyme solution. Corrections were made to compensate for protein dilution. The experiments were performed in 20 mM Hepes, pH 8.0, 0.1 mM EDTA, and 5 mM DTT. When the experiments were done in the presence of Mg^{2+} ions, the concentration of the cation was 10 mM. The Pfk-2 concentration was between 40 and 80 μg/mL. The data analysis was carried out with the Spectra Calc program (Galactica Corp.).

The fractional saturation binding by either ligand was determined from the intensity variation with free ligand concentration by calculating the quantity $(F^0 - F)/(F^0 - F^\infty)$, where F^0 represents the emission intensity in the absence of ligand, F^∞ is the emission intensity at saturating concentration of ligand, and F is the intensity after the addition of a given concentration of ligand.

Acrylamide quenching experiments were performed in 20 mM Hepes, 0.1 mM EDTA, and 5 mM DTT, pH 8.0, with an acrylamide stock solution of 5 M. The quenching data were analyzed by using the Stern−Volmer equation to obtain the respective constants (Stern−Volmer quenching constant and bimolecular quenching constant).

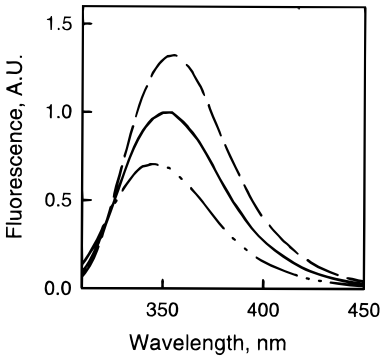

FIGURE 1: Fluorescence emission spectra of Pfk-2 in the absence and presence of ligands. Fluorescence spectra were measured at room temperature in 20 mM Hepes, pH 8.0, 0.1 mM EDTA, and 5 mM DTT. Excitation wavelength was set to 295 nm. (—) Pfk-2 in the absence of ligands; (- - -) Pfk-2 in the presence of 0.3 mM fructose-6-P; (—••—) Pfk-2 in the presence of 0.4 mM $MgATP^{2-}$. Fluorescence is expressed in arbitrary units relative to that of the free protein, taken as 1.0.

Lifetime Determinations. Tryptophan lifetime measurements were performed with a multifrequency cross-correlation phase and modulation fluorometer that uses the harmonic content of a high repetition rate, mode-locked Nd−YAG laser. This laser is used to synchronously pump a dye laser whose pulse train frequency is doubled with an angle-tuned frequency doubler (*16*). The excitation wavelength was 295 nm, and the emission was observed through a long-wavelength-pass filter (WG 320) with a cutoff at 320 nm. Lifetime studies were performed in a regular cuvette with *p*-terphenyl in cyclohexane in the reference cell. The lifetime values were obtained from the phase and modulation data by using the program Global Unlimited (Laboratory for Fluorescence Dynamics, University of Illinois, Urbana, IL). The data were analyzed with one, two, and three components for the decay of the tryptophan fluorescence. The best model was chosen by evaluating the goodness of the fit using the χ^2 values and the residuals between the modeled and the experimental data.

RESULTS

Effect of Ligands on the Intrinsic Fluorescent Spectrum of Pfk-2 from E. coli. The fluorescent characteristics of tryptophan residues depend strongly on the polarity of the microenviroment and thus provide a sensitive probe for the conformational state of the protein. Figure 1 shows the fluorescence spectrum of native Pfk-2 under several conditions. In the absence of ligands Pfk-2 has a emission maximum at 352 nm, which reflects a high degree of exposure of this residue to the solvent, since this emission maximum is characteristic of a tryptophan residue fully exposed to water (*18*). Upon addition of saturating concentrations of fructose-6-P (0.3 mM), the fluorescence intensity increases 30% with respect to that of the enzyme in the absence of ligands.

$MgATP^{2-}$ also modifies the intrinsic fluorescence of Pfk-2. Saturating concentrations of this ligand (0.4 mM) causes a quenching of fluorescence of about 30% of its initial value and the emission maximum is shifted to 345 nm, indicating less exposure to the solvent of the tryptophan residue in this enzyme−ligand complex.

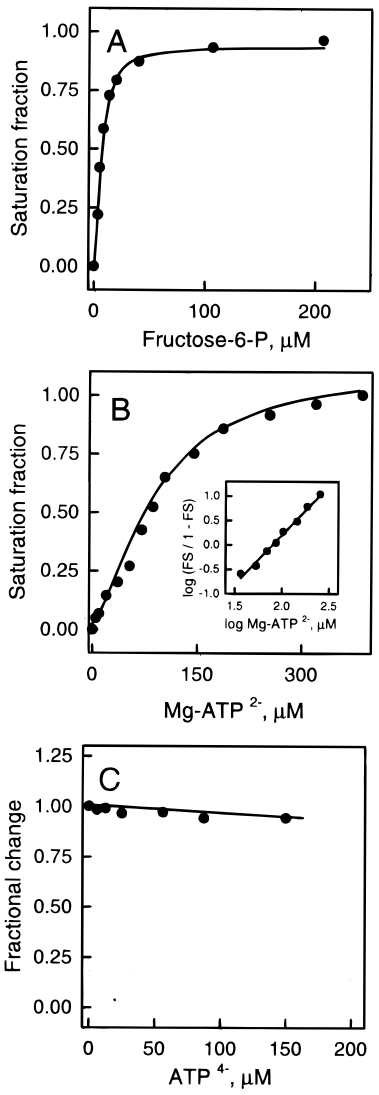

FIGURE 2: Effect of fructose-6-P, $MgATP^{2-}$, and ATP^{4-} concentration on the intrinsic fluorescence of Pfk-2. (A) Binding of fructose-6-P to Pfk-2 as followed by the increase in intrinsic fluorescence intensity. (B) Binding of $MgATP^{2-}$ to Pfk-2 as followed by the concomitant decrease in intrinsic Pfk-2 fluorescence. Percent saturation in panels A and B was calculated as described in the text. The inset in panel B shows a Hill plot of the data. (C) Effect of different concentrations of ATP^{4-} on the intrinsic fluorescence of Pfk-2. Mg ions were omitted from the buffer. Fluorescence is expressed as fractional change.

Fructose-6-P and ATP Binding to the Free Enzyme. The increase in fluorescence induced by fructose-6-P made possible the titration of Pfk-2 with this ligand. Figure 2A shows that the fluorescence emitted at 352 nm by Pfk-2 increases with a hyperbolic dependence on the concentration of fructose-6-P with a K_d value of 6 μM. The fluorescence is increased by about 30% at saturating concentrations of this substrate.

The fluorescence of Trp88 was also modified upon binding of $MgATP^{2-}$. As shown in Figure 2B, the fluorescence response of Pfk-2 to different $MgATP^{2-}$ concentrations is sigmoidal with a K_d of 80 μM and a Hill coefficient of approximately 2 (inset, Figure 2B).

Considering that no binding of $MgATP^{2-}$ to Pfk-2 should be expected in the absence of fructose-6-P, we next investigated the effect of ATP^{4-}, a compound that binds only to the catalytic site, on the fluorescence response of the enzyme. As shown in Figure 2C, this compound did not produce significant changes in the fluorescence properties of Pfk-2 at concentrations between 0 and 0.15 mM. Also, the effect of a nonhydrolyzable analogue of ATP, AMP-PNP, was studied. This compound behaves as a competitive inhibitor of Pfk-2 with respect to the substrate $MgATP^{2-}$ (K_i of 0.25 mM) and does not affect the fluorescence spectrum of the enzyme in the presence of Mg^{2+} (not shown).

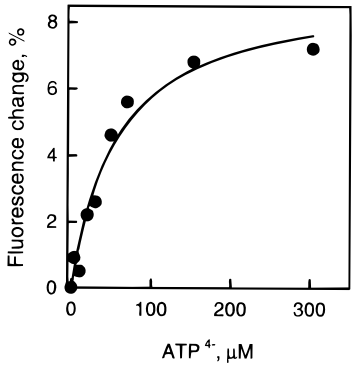

FIGURE 3: Effect of ATP^{4-} concentration on the intrinsic fluorescence of the Pfk-2−fructose-6-P complex. Binding of ATP^{4-} was followed by the increase in intrinsic fluorescence intensity of Pfk-2 in the presence of 0.1 mM fructose-6-P. The curve is fitted to the hyperbola equation. The fluorescence change is expressed as percent of the control fluorescence in the presence of 0.1 mM fructose-6-P and in the absence of ATP^{4-}.

ATP^{4-} Binding to the Enzyme−Fructose-6-P Complex. Since ATP^{4-} is not able to bind to the free enzyme in the absence of fructose-6-P, it should bind in its presence. Figure 3 shows the effect of increasing concentrations of ATP^{4-} upon the fluorescence of Pfk-2 in the presence of 100 μM fructose-6-P. The nucleotide produces a hyperbolic response with a maximal fluorescence increase of 8% and a K_d of 56 μM.

Effect of the Fructose-6-P Concentration on the Fluorescence of the Enzyme−$MgATP^{2-}$ Complex. Kinetic studies demonstrated that the inhibition of Pfk-2 by $MgATP^{2-}$ was observed at low fructose-6-P concentrations (0.1 mM), while the inhibitory effect disappears at higher concentrations of the substrate (*3*). Also, the $MgATP^{2-}$ binding to Pfk-2 has been correlated with a dimer−tetramer association process, in which the effectiveness of this ligand in inducing the aggregation closely parallels its kinetic inhibitory behavior (*6*). Since the binding of $MgATP^{2-}$ causes a fluorescence quenching of the tryptophan emission, we decided to assess the effect of fructose-6-P on this response. Figure 4 shows the fluorescence changes induced by the addition of fructose-6-P to Pfk-2 in the presence of 0.4 mM $MgATP^{2-}$. An increase in the fructose-6-P concentration relieves the quenching produced by the nucleotide−Mg complex. At a concentration of fructose-6-P of about 70 μM, the quenching effect is completely overcome and the fluorescence obtained corresponds to that of the enzyme in the absence of ligands (Figure 4, dotted line). Higher concentrations of fructose-6-P produce the typical increase in fluorescence obtained for this substrate. Although the products of the reaction are in the medium, neither of them (ADP or fructose-1,6-bisP) produces any effect on the fluorescence spectrum of Pfk-2 (not shown). The gradual increase in fluorescence with

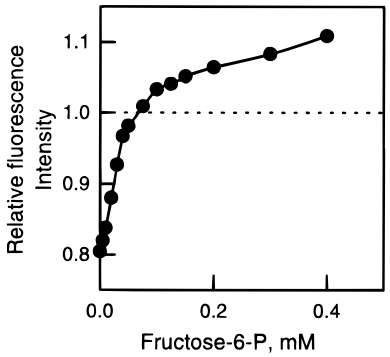

FIGURE 4: Fluorescence change of Pfk-2 upon addition of fructose-6-P to the enzyme−$MgATP^{2-}$ complex. The effect of fructose-6-P concentration was followed by the increase in intrinsic fluorescence intensity of Pfk-2 in the presence of 0.4 mM $MgATP^{2-}$. Fluorescence is expressed relative to that of the Pfk-2−$MgATP^{2-}$ complex, taken as 1.0 (dotted line).

Table 1: Tryptophan Lifetime Determinations for Pfk-2 in the Absence and Presence of Saturating Concentrations of Fructose-6-P and $MgATP^{2-}$ [a]

ligand added[b]	τ_{long} (ns)	τ_{short} (ns)	$\langle\tau\rangle$ (ns)
none	8.6 ± 0.1	2.8 ± 0.3	7.7 ± 0.3
fructose-6-P	9.3 ± 0.5	3.6 ± 0.3	8.2 ± 0.2
$MgATP^{2-}$	8.8 ± 0.3	3.1 ± 0.2	7.7 ± 0.1

[a] The determinations were carried out as described in Materials and Methods. [b] The ligand concentrations were fructose-6-P, 0.4 mM, and $MgATP^{2-}$, 0.4 mM.

increasing concentrations of fructose-6-P is the result of ATP depletion as the reaction proceeds, which implies ATP hydrolysis promoted by binding of fructose-6-P to the catalytic site of the quenched state. This response is cooperative with a Hill coefficient of 1.5, and the fructose-6-P concentration at which the quenching effect is completely overcome is approximately 10 times higher than the binding constant obtained for fructose-6-P binding to the free enzyme.

Determination of Tryptophan Lifetime Components for Pfk-2. The intensity average lifetimes, as well as the lifetime of the long and short components for Pfk-2, in the absence and presence of ligands, were determined. In all cases, the best fit of the phase and modulation data was obtained with a model of two discrete exponential decays. The χ^2 values for models of one, two, and three components were 6.3, 0.2, and 0.2 for the enzyme in the absence of ligands; 110, 4.3, and 4.6 in the presence of $MgATP^{2-}$; and 4.6, 0.3, and 0.2 in the presence of fructose-6-P, respectively. The majority of the fluorescence intensity (>80%) corresponds to a longer component, which is around 8.0 ns. The lifetime of the short component is about 3.0 ns. Neither of these components exhibited any significant variation with the state of ligation of the enzyme, whether in the presence of fructose-6-P or $MgATP^{2-}$ (Table 1).

Effect of Fructose-6-P and $MgATP^{2-}$ on the Fluorescence Polarization of Pfk-2. The pattern of ligand-induced changes of fluorescence polarization of the intrinsic tryptophan of Pfk-2 was very similar to that described for the changes in intensity. Fructose-6-P and $MgATP^{2-}$ cause divergent changes to the intrinsic polarization; fructose-6-P causes a substantial increase and $MgATP^{2-}$ a decrease in this property (Figure 5).

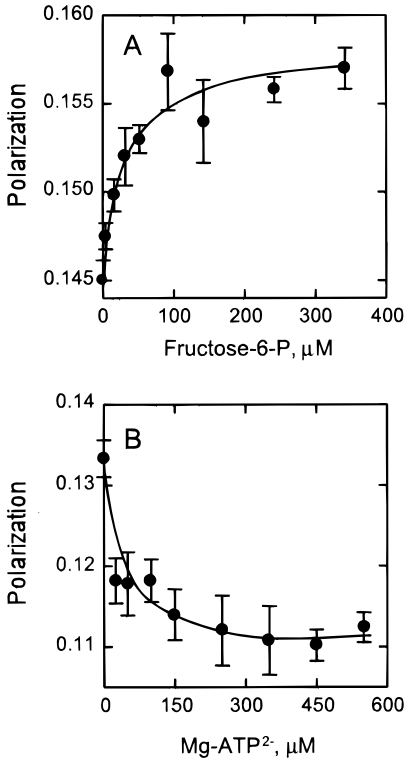

FIGURE 5: Fluorescence polarization changes of Pfk-2 upon binding of ligands. Pfk-2 was titrated with either fructose-6-P (A) or $MgATP^{2-}$ (B). Polarization determinations were made using an L format and an excitation wavelength of 295 nm. Results are expressed as the means ± SD of five determinations.

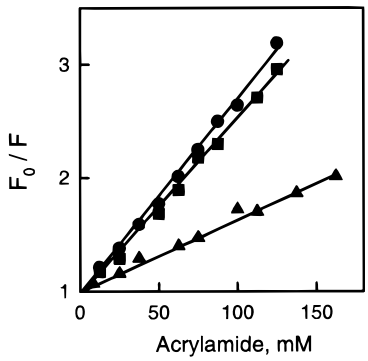

FIGURE 6: Stern−Volmer plots for acrylamide for different Pfk-2-ligand complexes. (●) Free enzyme; (■) Pfk-2 in the presence of 0.28 mM fructose-6-P; (▲) Pfk-2 in the presence of 0.4 mM $MgATP^{2-}$.

Steady-State Quenching Experiments on Pfk-2. Quenching experiments are commonly performed with tryptophan to determine the extent of its exposure to the solvent. The quenching ability of neutral acrylamide was used for different enzyme−ligand complexes. Care was exercised with acrylamide since it is known to exhibit static quenching (ground-state formation of a nonfluorescent complex) at higher concentrations. The presence of such quenching is characterized by an upward curvature of the Stern−Volmer plot (*17*). Figure 6 shows the Stern−Volmer plots for acrylamide for the different enzyme complexes of Pfk-2. The lines in Figure 6 were obtained by fitting the data to the Stern−Volmer equation, $F_0/F = 1 + K_{sv}[Q]$, where F_0 and F are the fluorescence intensities in the absence and presence of the quenching agent, respectively. K_{sv} is the Stern−Volmer

Table 2: Stern−Volmer and Bimolecular Quenching Constants for Trp88 in Pfk-2 in the Absence and Presence of Saturating Concentrations of Fructose-6-P and $MgATP^{2-}$

ligand added[a]	K_{sv} (M^{-1})	k_q ($\times 10^9$ M^{-1} s^{-1})
none	16.8	2.3
fructose-6-P	15.2	1.9
$MgATP^{2-}$	6.4	0.83

[a] The ligand concentrations were fructose-6-P, 0.4 mM, and $MgATP^{2-}$, 0.4 mM.

quenching constant and [Q] is the concentration of the quencher. For the concentrations used in this experiment, the Stern−Volmer plots show a linear relationship between fluorescence intensity and quenching reagent concentration, which is generally indicative of a single class of fluorophores all equally accessible to the quencher and suggests the absence of a static component to the quenching (*18*). The calculated Stern−Volmer and bimolecular quenching constants (k_q) for Pfk-2 under several conditions are summarized in Table 2. The k_q obtained for Trp in the free enzyme and in the enzyme−fructose-6-P complex is 2.6 times larger than that in the enzyme−$MgATP^{2-}$ complex. The magnitude of k_q for the enzyme−$MgATP^{2-}$ complex clearly demonstrate that the Trp in this complex is exposed to a lesser extent to the solvent than in the free enzyme or in the enzyme−fructose-6-P complex.

DISCUSSION

Tryptophan residues can often be used as fluorescent probes to investigate the dynamic nature of proteins. For *E. coli* Pfk-2, binding of fructose-6-P causes a hyperbolic increase in the intrinsic fluorescence with a K_d value of 6 μM, which is approximately 5 times lower than the K_m value obtained from kinetic measurements. This result is in agreement with the kinetic mechanism of Pfk-2 in which fructose-6-P is the first substrate to bind to the enzyme in a hyperbolic fashion (*5*). In the case of *E. coli* Pfk-1, different K_d values were found for the dissociation constant of the same ligand in equilibrium dialysis experiments and fluorescence measurements (*7*, *9*, *10*). Deville-Bonne and Garel (*9*) suggest that the lower values determined by fluorescence may be explained if changes in fluorescence intensity are not proportional to the degree of saturation.

On the other hand, the discrepancy found between the activity and fluorescent measurements of Pfk-2 might be due to the presence of saturating concentrations of fructose-6-P when the K_m for ATP was determined by activity measurements. Kinetic measurements showed that $MgATP^{2-}$ binds to the enzyme−fructose-6-P complex in a hyperbolic fashion with a K_m of 20 μM, while through steady-state intrinsic fluorescence quenching it was shown that the binding of $MgATP^{2-}$ to the free enzyme is cooperative ($n_h = 2$) with a K_d value of 80 μM (Figure 2). This curve and the dissociation constant obtained are very similar to those obtained for the effect of $MgATP^{2-}$ on the aggregation state of Pfk-2, where the middle point for the dimer−tetramer transition is close to 70 μM (*6*).

$MgATP^{2-}$ and ATP^{4-} present very similar affinities for the active site of Pfk-2 but produce different effects on the fluorescence and kinetic characteristics of the enzyme. The kinetic and fluorescence data can be reconciled if $MgATP^{2-}$ acted as an allosteric inhibitor. Pfk-2 presents a compulsory order kinetic mechanism with fructose-6-P being the first substrate to bind to the enzyme (5), which rules out binding of $MgATP^{2-}$ in the absence of fructose-6-P. However, binding of this ligand to Pfk-2 in the absence of the sugar substrate promotes a quenching of the intrinsic fluorescence response in a cooperative fashion. On the other hand, compounds known to bind to the active site of Pfk-2, such as ATP^{4-}, are not able to bind to the free enzyme but do bind to the enzyme−fructose-6-P complex in a hyperbolic fashion, in accordance with the kinetic data. Also, AMP-PNP, a competitive inhibitor of $MgATP^{2-}$ at the catalytic site, did not produce any changes in the fluorescence spectrum of Pfk-2. Thus, it can be concluded that binding of $MgATP^{2-}$ to the free enzyme occurs at an allosteric site, which is rather specific for this ligand, compared to the catalytic site, which can accept other nucleotides. Also, the kinetic data show that $MgATP^{2-}$ inhibits the enzyme activity at low concentrations of fructose-6-P and promotes the aggregation of Pfk-2, while at high concentrations of fructose-6-P no inhibition is observed and the enzyme is present as a dimer. If the fluorescence quenching produced by $MgATP^{2-}$ is a consequence of its binding to the allosteric site of the enzyme, this compound probably binds to the tetrameric form of Pfk-2 and, therefore, the inhibited one. Then, one could expect that an increase in the fructose-6-P concentration would relieve the fluorescence quenching produced by $MgATP^{2-}$, as already shown in Figure 4.

Inasmuch as fluorescence intensity is an index of protein conformation, only two states seem possible for the binary complex between Pfk-2 and one of its ligands: one conformational state has a high fluorescence (130% as compared to free Pfk-2) and the other has a low fluorescence (70% that of unligated Pfk-2). The high fluorescence state favored by the binding of fructose-6-P probably corresponds to the active conformation. The low fluorescence state favored by the binding of $MgATP^{2-}$ could correspond to the inhibited one, probably the tetramer. A similar situation was described for the Pfk-1 isozyme, where the high fluorescence state is favored by the binding of P-enolpyruvate and probably corresponds to the conformation described by Schirmer and Evans (*19*) for the binary complex between Pfk-1 and P-enolpyruvate. The low fluorescence state, favored by the binding of ADP, could correspond to the conformation described by Shirakibara and Evans (*20*) for the ternary complex between Pfk-1, ADP, and fructose-1,6-bisP. These states could therefore correspond to the T and R states of the concerted model (*2*).

Since three fluorescence states of Pfk-2 were observed, one with a high quantum yield (Pfk-2 saturated with fructose-6-P), another with an intermediate emission (Pfk-2 without ligands), and a third with a low quantum yield state (Pfk-2 in the presence of $MgATP^{2-}$), tryptophan lifetimes were measured to determine the origin of these differences. The lifetime values show that they are independent of the state of ligation of the enzyme and that the different Pfk-2 complexes have almost the same lifetime values for the intrinsic probe (Table 1). This indicates that the source of enhancement or quenching may have its origin in the formation of static complexes, where the quenched molecules might have a large radiationless rate constant or a very small

emission rate, which results in a very long lifetime component (18).

Changes in the fluorescence polarization may be due to the overall motions of the particle as a rigid or quasirigid body or to local rotations that involve the fluorophore alone. However, changes in the polarization of the intrinsic protein fluorescence, which involves fluorophores with lifetimes between 2 and 6 ns, cannot usually be relied on to give an estimate of the change in volume of the protein. In this case the changes in polarization are probably achieved either through alterations in the local motions of the fluorophore in the protein or by changing the fluorescence lifetime. Since the lifetime of the tryptophan residue in Pfk-2 does not vary upon the ligation state of the enzyme (Table 1), the most plausible explanation for this polarization change is that it is due to differences in the local mobility of the Trp in both enzyme−ligand complexes as compared to the free enzyme. Since an increase in fluorescence polarization is observed upon fructose-6-P binding, the conformational change produced by this ligand would result in a smaller degree of local rotational freedom of the Trp in the protein, opposite to the effect observed with $MgATP^{2-}$.

Solute quenching of protein fluorescence is a technique that can yield information regarding the solvent exposure of the intrinsic or extrinsic fluorophore. It is a dynamic method, since the presence of solutes alters the fluorescence properties of the protein. One of the most attractive features of acrylamide as a quencher has been the fact that it does not interact specifically with proteins, and due to its high hydrophilicity, it behaves as a collisional quencher of probes located at the surface of proteins (*17*). In the presence of acrylamide, Pfk-2 shows a linear Stern−Volmer plot, indicating the occurrence of collisional quenching process only, the same as for the Pfk-2−substrate complexes. The K_{sv} for acrylamide quenching for the Pfk-2−$MgATP^{2-}$ complex (6.4 M^{-1}) is much lower than that of the free enzyme (16.8 M^{-1}) or the Pfk-2−fructose-6-P complex (15.2 M^{-1}), indicating that tryptophan accessibility to the solvent is reduced in this complex, perhaps due to tetramer formation. Also, the high K_{sv} values found for the free enzyme and the Pfk-2−fructose-6-P complex indicate a high degree of solvent accessibility of the tryptophans under these conditions, since a value of 11.4 M^{-1} has been reported for unfolded rhodanese (*21*). Iodide quenching Stern−Volmer analysis of the tryptophan in Pfk-1 showed significant changes in the accessibility to the solvent as a function of the ligation state of the enzyme. The tryptophan in Pfk-1 bound to fructose-6-P is approximately 3 times more accessible than that of the unligated enzyme, while the tryptophan of the ternary complex of MgADP−PFK−fructose-6-P is 2-fold more readily quenched than that of the free enzyme (*22*).

A regulatory role for $MgATP^{2-}$ has also been proposed for *E. coli* Pfk-1. Berger and Evans (*7*) demonstrated that ATP or AMP-PNP increase the intrinsic fluorescence of Pfk-1 slightly and that both cause inhibition of the fluorescence change at low concentrations of fructose-6-P. Although it is not clear from the structure of the active site and from the model for the R state that ATP is acting through a site distinct from the active site, some features of the proposed model lead these authors to suggest that $MgATP^{2-}$ could bind to a second allosteric site. The antagonistic binding of fructose-6-P and $MgATP^{2-}$ to Pfk-1 has been extensively studied. Deville-Bonne and Garel (*9*) suggest that both substrates of Pfk-1 are binding antagonists, since ATP binds to the T state and fructose-6-P to the R state, which in turn correspond to the high and low fluorescence states, respectively. This antagonism is also partially detected by activity measurements, since the apparent K_m for one substrate increases with the concentration of the other substrate (*8*, *11*). Deville-Bonne et al. (*8*) concluded that $MgATP^{2-}$ and fructose-6-P bind in a rapid-equilibrium random manner with mutual antagonism arising when both substrates bind within each single active site. However, Johnson and Reinhart (*10*) concluded, through a linked-function analysis of the interactions of $MgATP^{2-}$ and fructose-6-P, that no additional binding interactions, other than multiple active sites present in the oligomer, are necessary to explain this behavior. Moreover, using the derived equations these authors can distinguish intra-active-site and inter-active-site antagonism between fructose-6-P and $MgATP^{2-}$. This analysis allows one to conclude that the substrate antagonism exhibited by Pfk-1 is at least partially due to an influence between active sites, contrary to the conclusions of Deville-Bonne et al. (*8*). Also, PFK from *Bacillus stearothermophilus* shows inhibition by $MgATP^{2-}$, which is proposed to result from both abortive binding of $MgATP^{2-}$ in the fructose-6-P site and reaction flux through a kinetically disfavored pathway in a random mechanism (*23*).

On the other hand, studies of the binding of nucleotides to rabbit muscle PFK by changes in intrinsic protein fluorescence demonstrate that quenching of fluorescence produced by $MgATP^{2-}$ is associated with binding to an inhibitory site (*24*).

The results presented in this paper, together with the kinetic data reported earlier, support a regulatory role for $MgATP^{2-}$ in the Pfk-2 enzyme, through its binding to an allosteric site. This allosteric site would in turn be related to the inhibition of enzyme activity and tetramer formation. The physiological consequences for the absence of this regulatory property have been recently reported in studies with a mutated form of the enzyme (*25*). Structural studies are in progress in order to establish the residues present in this allosteric site.

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