Site-Directed Mutagenesis Identifies Catalytic Residues in the Active Site of *Escherichia coli* Phosphofructokinase†

Stuart A. Berger‡ and Philip R. Evans*

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, England

Received December 19, 1991; Revised Manuscript Received June 12, 1992

ABSTRACT: Six active site mutants of *Escherichia coli* phosphofructokinase have been constructed and characterized using steady-state kinetics. All but one of the mutants (ES222) have significantly lower maximal activity, implicating these residues in the catalytic process. Replacement of Asp127, the key catalytic residue in the forward reaction with Glu, results in an enzyme with wild-type cooperative and allosteric behavior but severely decreased Fru6P binding. Replacement of the same residue with Tyr abolishes cooperativity while retaining sensitivity to allosteric inhibition and activation. Thus, this mutant has uncoupled homotropic from heterotropic allostery. Mutation of Asp103 to Ala results in an enzyme which retains wild-type Fru6P-binding characteristics with reduced activity. GDP, which allosterically activates the wild-type enzyme, acts as a mixed inhibitor for this mutant. Mutation of Thr125 to Ala and Asp129 to Ser produces mutants with impaired Fru6P binding and decreased cooperativity. In the presence of the activator GDP, both these mutants display apparent negative cooperativity. In addition, ATP binding is now allosterically altered by GDP. These results extend the number of active site residues known to participate in the catalytic process and help to define the mechanisms behind catalysis and homotropic and heterotropic allostery.

Phosphofructokinase catalyzes the phosphorylation of fructose 6-phosphate (Fru6P) and is the key control point in the glycolytic pathway (Uyeda, 1979). Although PFKs from many organisms often display very complex control, the prokaryotic enzymes tend to be somewhat simpler. For instance, the enzyme from *E. coli* (PFK-1; EC 2.7.1.11) is a tetramer and displays cooperative kinetics with respect to Fru6P, hyperbolic kinetics with respect to ATP, allosteric activation by the purine nucleoside diphosphates ADP and GDP, and inhibition by phosphoenolpyruvate (PEP). The kinetic properties of this enzyme fit reasonably well to a simple two-state model (Blangy et al., 1968), in which, in the absence of Fru6P or in the presence of PEP, PFK exists primarily in an active ("T") state with low affinity for Fru6P. With increasing concentrations of Fru6P or in the presence of ADP or GDP, the active ("R") state with high affinity for Fru6P becomes stabilized, leading to increased binding and catalysis. However, recent data suggest that this model is too simplistic. For instance, although the principal effect of the activator GDP is to increase the affinity of PFK for Fru6P, it also increases the V_{max} (Deville-Bonne et al., 1991a; Table I). More seriously, the binding of Fru6P on its own is observed to be hyperbolic, with 2 sites per tetramer, but to become sigmoidal on addition of stable ATP analogues (Deville-Bonne & Garel, 1992; Berger & Evans, 1991). This suggests that the low-affinity T state is induced by the binding of ATP, while the free enzyme is in an R-state conformation, consistent with the observed R-state conformation of unliganded *Escherichia coli* PFK in the crystal (Rypniewski & Evans, 1989). Binding of ATP also inhibits binding of Fru6P within the R-state conformation, as shown by the substrate antagonism in the presence of the activator GDP (Deville-Bonne et al., 1991b), but this effect cannot account for the change in cooperativity. The full relationship between binding and observed steady-state kinetics remains unknown.

Structural (Evans & Hudson, 1979; Evans et al., 1981; Shirakihara & Evans, 1988), kinetic (Blangy et al., 1968; Hellinga & Evans, 1987), and stereochemical (Jarvest et al., 1981) evidence suggests that the catalytic mechanism of the phosphoryl transfer involves an in-line nucleophilic attack by the 1-hydroxyl of Fru6P on the γ-phosphate of ATP. A model for the transition state is shown in Figure 1, based on crystal structures of complexes of *E. coli* PFK with the reaction products Fru1,6P_2 and ADP/Mg^{2+} (Shirakihara & Evans, 1988), and with the substrate Fru6P and the ATP analogue AMPPNP (T. Schirmer, unpublished). Asp127 probably acts as a base, increasing the nucleophilicity of the 1-hydroxyl of Fru6P by abstracting its proton. The pentacoordinated γ-phosphate transition state may be stabilized by several groups including the side chains of Arg72, Arg171, Gly11, and Thr125, and by the Mg^{2+} cofactor. Both Asp103 and Asp129 stabilize the position of the Mg^{2+} ion: Asp129 binds to the Mg^{2+} ion through two water molecules.

Mutants of PFK (constructed using site-directed mutagenesis) have been particularly helpful in identifying the role of certain active site residues in this process. In particular, Asp127 has been confirmed as the key catalytic residue in the kinase reaction probably acting as a base catalyst (Hellinga & Evans, 1987): mutation of this residue to Ser decreases the catalytic activity of the enzyme by 15 000–18 000 compared to wild type (Hellinga & Evans, 1987; S. A. Berger unpublished). Arg171 plays a minor role in catalysis: mutation of this residue to Ser decreases catalysis 3-fold (Hellinga & Evans, 1987). In the R state, Arg72 bridges the two substrates, while in the T state, it participates in a salt bridge across the interface with Glu241 (Schirmer & Evans, 1990): truncation of this residue to Ser results in an enzyme with reduced catalytic ability, slightly reduced Fru6P binding, and reduced cooperativity (Berger & Evans, 1990). Arg162 and Arg243 extend into the active site from the adjacent subunit. In the R state, both of these residues interact with the 6-phosphate of Fru6P.

† S.A.B. was supported by a fellowship from the MRC of Canada.

* Corresponding author [telephone, (0223) 248011; fax, (0223) 213556].

‡ Present address: Biomedical Research Centre, 2222 Health Science Mall, University of British Columbia, Vancouver, BC, V6T 1W5 Canada.

0006-2960/92/0431-9237$03.00/0

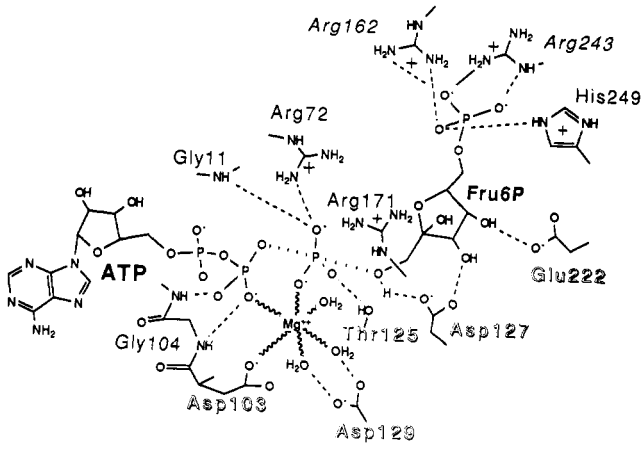

FIGURE 1: Schematic diagram of selected active site residues and the proposed transition state in *E. coli* PFK. Residues Arg162 and Arg243 (labeled in italic) originate from the adjacent subunit. Highlighted labels indicate residues mutated in this study.

Truncation of these residues to Ser results in enzymes with decreased Fru6P binding ability and reduced cooperativity but little change in catalytic ability (Berger & Evans, 1990). These results identify Arg72 as a participant in the differential stabilization of the transition state. They also identify these three arginines as being particularly important in transmitting cooperative and allosteric signals between subunits.

Other active site residues are also well-placed to participate in the catalytic process. In an attempt to further extend our understanding of the catalytic and allosteric mechanism of this enzyme, we have constructed and characterized additional mutations in the active site. Mutations in residues Asp103, Thr125, and Asp129 were constructed primarily to test their importance in catalysis; mutations in Asp127 and Glu222 were constructed in an attempt to make an enzyme with a low affinity for Fru6P.

MATERIALS AND METHODS

Site-Directed Mutagenesis. Site-directed mutagenesis was performed on pEMBL-based plasmid pHL1 (Lau et al., 1987). This vector consists of the PFK-1 gene from *E. coli* cloned into the LacZ region of pEMBL8$^+$. Single-stranded DNA was produced as described by Dente et al. (1983) with some modifications. The double-stranded form of the vector was transformed into HB101 F′::Tn5 cells (which contain the F′ factor linked to the Kan^R gene; a gift from F. K.-T. Lau, Imperial College) using the procedure of Hanahan (1983). Individual colonies were picked and grown as overnight cultures in the presence of ampicillin and kanamycin (both at concentrations of 50 μg/mL) in 2× TY broth. These cultures were diluted 1:100 and allowed to grow for 3 h at 37 °C. Approximately 7 × 10^9 R408 helper phage/mL of culture was added, and the tubes were incubated for another $4^1/_2$ h. The cells were then pelleted, the supernatant was collected, and the phase was precipitated in 2.5 M NaCl and 20% (w/v) PEG 6000. The DNA was separated from phage proteins by extensive vortexing in 50% phenol and then precipitated with sodium acetate and ethanol.

Mutagenesis was performed by annealing this ss DNA with appropriate oligonucleotides and extending and ligating as described (Zoller & Smith, 1983). This DNA was then transformed into HB101 F′::Tn5 cells, and colonies were screened for the presence of the plasmid using hybridization. As many of these colonies contained mixtures of wild-type and mutant plasmid, positive colonies were picked, ds DNA was extracted and transformed into fresh cells, and a second round of screening was performed. Positives on the second round were screened by dideoxy-sequencing (Sanger et al., 1977) using appropriate primers. Efficiencies of mutagenesis varied from 5% to 30% of the colonies screened. All mutant genes were completely sequenced to verify that no spurious mutations had been introduced.

Expression and Purification. Double-stranded plasmid DNA containing either wild-type or mutant PFK was transformed into DF1020 cells (which are deleted for both *E. coli* PFK genes), and individual colonies were grown overnight. These cultures were then diluted 1:250 in the presence of 70 μg/mL IPTG and grown for 24 h. Cells were pelleted, resuspended in 1/40th 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 at 15 000 rpm (Sorvall) for 20 min followed by a second spin at 40 000 rpm (Ti–70, Beckman) for 90 min. This supernatant was loaded directly onto a Cibacron Blue-A column (Amicon) (or a Red-A column in the case of DA103) as described (Kotlarz & Buc, 1977). The column was washed with TE and 1 M NaCl in TE, and PFK was eluted with 2–4 mM ATP and 10 mM $MgCl_2$ in TE. Fractions positive in the Bradford protein assay (Bio-Rad) were pooled, concentrated in an Amicon pressure cell, and stored as a 55% ammonium sulfate precipitate. As *E. coli* PFK is sensitive to air oxidation (Banas et al., 1988), all PFK solutions were stored in 10 mM DTT under argon. This extended the lifetime of the stored enzyme considerably.

Before use, aliquots of PFK were desalted on an S-200 Sephacryl column (Pharmacia). Protein concentration was measured using absorbance (specific absorbance ϵ_{278} = 0.6 cm^2 mg^{-1}).

Kinetic Assays. Forward reaction enzyme activity was measured at 25 °C, in 50 mM Tris, pH 8.0, 10 mM DTT, 10 mM $MgCl_2$, and 10 mM NH_4Cl, by coupling Fru1,6P_2 production to the oxidation of NADH using aldolase (20 μg/mL), triosephosphate isomerase (10 μg/mL), and glycerol-3-phosphate dehydrogenase (10 μg/mL) as described (Kotlarz & Buc, 1977). ADP produced by the reaction was regenerated to ATP using creatine phosphate (1 mM) and creatine kinase (10 μg/mL). Reverse reaction measurements were as for the forward reaction, except the production of Fru6P was coupled to the reduction of NADP with glucose-6-phosphate isomerase (10 μg/mL) and glucose-6-phosphate dehydrogenase (10 μg/mL) (Hellinga & Evans, 1987). ATP was regenerated to ADP with glycerol (1 mM) and glycerol kinase (20 μg/mL). All coupling enzymes and substrates were from Boehringer Mannheim. Coupling enzymes were purified on a Sephadex G-50 column before use. Steady-state initial velocities were measured in a Beckman DU-65 6-cell spectrophotometer by following the change in NADH absorbance at 340 nm. Kinetic parameters were obtained by fitting data either to the Michaelis–Menten equation [$V = V_{max}[S]/(K_m + [S])$] for hyperbolic kinetics or to the Hill equation [$V = V_{max}[S]^n/(S_{1/2}{}^n + [S]^n)$] for sigmoidal kinetics using a nonlinear least-squares fitting routine (S.A.B.).

RESULTS

Production and Expression of Mutants. The mutants described in this paper are Asp103→Ala (DA103), Thr125→Ala (TA125), Asp127→Glu (DE127), Asp127→Tyr (DY127), Asp129→Ser (DS129), and Glu222→Ser (ES222). Most of the mutations were side-chain deletions to Ala or Ser. Asp103 and Thr125 were replaced by Ala because they are located

Table I: Steady-State Kinetic Parameters of Wild-Type and Mutant PFKs[a]

	forward reaction							reverse reaction		
	+GDP			-GDP						
enzyme	V_{max} (s^{-1})	$S_{1/2}$ (μM)	n_H	V_{max} (s^{-1})	$S_{1/2}$ (μM)	n_H	$K_{m[ATP]}$ (μM)	V_{max} (s^{-1})	$K_{m[Fru1,6P_2]}$ (μM)	$K_{m[ADP]}$ (μM)
w.t.	134	30	(h)	124	540	4.0	63	22	1900	52
DA103	4.8	28	(h)	6.7	386	3.7	30	1.9	4900	322
TA125	0.15	9600	0.55	0.12	850	(h)	32	0.007	15300	100
DE127	1.1	9740	(h)	1.1	87000	3.6	6	nd	nd	nd
DY127	0.14	52	(h)	0.1	4430	(h)	19	nd	nd	nd
DS129	0.14	357	0.8	0.31	2300	2.7	26	0.03	6600	387
ES222	120	24	(h)	85	226	3.7	66	13	2500	81

[a] w.t.: wild type. $S_{1/2}$: Fru6P concentration at half-maximal velocity. n_H: Hill constant. Forward reaction parameters were measured in the presence or absence of 1 mM GDP. Fru6P kinetics were measured in the presence of 1 mM ATP. (h) indicates that the kinetics are hyperbolic with respect to Fru6P ($n_H = 1.0 \pm 0.1$). $K_{m[ATP]}$ values were measured in the absence of GDP and in the presence of saturating values of Fru6P (for TA125, 2 mM Fru6P; for DE127, DY127, and DS129, 10 mM Fru6P; for wild type, DA103, and ES222, 1 mM Fru6P).

in relatively hydrophobic regions of the molecule. Asp129 and Glu222 are more exposed to solvent, so they were replaced with the more polar Ser. Mutants with Asp127 replaced with Glu or Tyr were designed to inhibit the binding of substrates by blocking the active site with a bulky group.

Expression of these mutants in DF1020 cells was relatively uniform. Protein yield was approximately 50–100 mg/L of culture. All mutants were purified using the same procedure as with the wild-type enzyme except for DA103, which was unable to be eluted from the Blue-A column. Instead, DA103 was eluted from a Red-A dye column with 2 mM ATP and 10 mM $MgCl_2$.

TA125. The side-chain hydroxyl group of Thr125 is hydrogen-bonded to the transferred phosphoryl group, both to the 1-phosphate of the product Fru1,6P_2 (Shirakihara & Evans, 1988) and to the γ-phosphate of the ATP analogue AMPPNP (Schirmer, unpublished). It is likely that this interaction stabilizes the pentacoordinate transition state, and this is supported by the large reduction in catalytic rate caused by mutation to alanine (V_{max} reduced by a factor of 900 in the forward reaction and by a factor of 3200 in the reverse reaction, Table I). Removal of this interaction might be expected to reduce the affinity of the enzyme for Fru1,6P_2 and for ATP, and indeed $K_{m[Fru1,6P_2]}$ is increased by a factor of 8, but there is little change in $K_{m[ATP]}$ (Table I).

The wild-type enzyme shows a highly sigmoidal dependence of rate on [Fru6P]. In contrast, mutant TA125 displays an approximately hyperbolic response ($n_H = 0.91 \pm 0.03$) with respect to Fru6P in the absence of effectors. In the presence of GDP the kinetics with respect to Fru6P display apparent negative cooperativity, as characterized by a Hill coefficient of 0.55 ± 0.03 (Table I, Figure 2). Both with and without GDP, the apparent affinity for Fru6P is reduced (half-saturation $S_{1/2}$ in the absence of GDP is larger than K_R for the wild type by a factor of about 28), despite there being no direct contact between Thr125 and Fru6P. The origin of the sigmoidal kinetics of the wild-type enzyme is not fully understood, but the observed hyperbolic binding of Fru6P in the absence of ATP (Berger & Evans, 1991; Devill-Bonne & Garel, 1992) indicated that it may arise from the antagonistic interaction between the two substrates, perhaps by ATP inducing the T state (see the Discussion). The TA125 mutation might interfere with this process, so that the loss of the interaction with ATP somehow leads to impaired binding of Fru6P.

In addition to its effects on Fru6P kinetics, GDP also affects the kinetics with respect to ATP. In the presence of nonsaturating concentrations of Fru6P, and with increasing concentrations of GDP, V_{max} for TA125 decreases with little

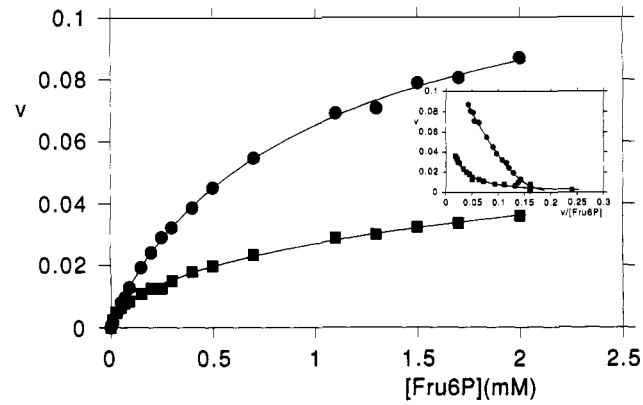

FIGURE 2: Steady-state kinetics of mutant TA125, showing hyperbolic dependence of rate on [Fru6P] in the absence of effectors (●) and negatively cooperative dependence in the presence of 1 mM GDP (■). Inset: Eadie–Hofstee representation of the same data, showing the curvature characteristic of negative cooperativity (■).

change in the $K_{m[ATP]}$ (Figure 5a). This pattern is characteristic of noncompetitive inhibition (Palmer, 1985) and suggests that this effect is mediated through the allosteric binding site.

DS129. Asp129 binds the essential Mg^{2+} ion indirectly through two water molecules, in both the substrate and product complexes (Figure 1). Mutation to serine causes a large reduction in catalytic rate, by a factor of 960 for the forward reaction and by a factor of 730 for the back-reaction (Table I). The correct positioning of the Mg^{2+} ion by the aspartate side chain is presumably critical for efficient catalysis. This mutation also impairs the apparent binding of Fru6P, although there is no direct contact. The half-saturation concentration of Fru6P, $S_{1/2[Fru6P]}$, is increased by about 4 from wild type, with a reduction in cooperativity. The corresponding K_R from fitting the data to the Monod–Wyman–Changeux equation is increased by a factor of 22 from the wild type. Addition of GDP activates as for wild type, but decreases V_{max} (Figure 3): like TA125, there is some evidence for negative cooperativity in the presence of GDP (as shown by the curved Eadie–Hofstee plot in Figure 3 and the calculated Hills coefficient of 0.8), but it is less pronounced than for TA125.

In the presence of nonsaturating concentrations of Fru6P, increasing concentrations of GDP also affect the kinetics with respect to ATP, causing decreases in both V_{max} and $K_{m[ATP]}$ (Figure 5b). This unusual pattern suggests that, like TA125, the inhibition mechanism is acting through the allosteric rather than the active site.

DA103. Asp103 binds the Mg^{2+} ion directly in both substrate and product complexes (Figure 1), and like Asp129, its mutation causes a reduction in catalytic rate, though

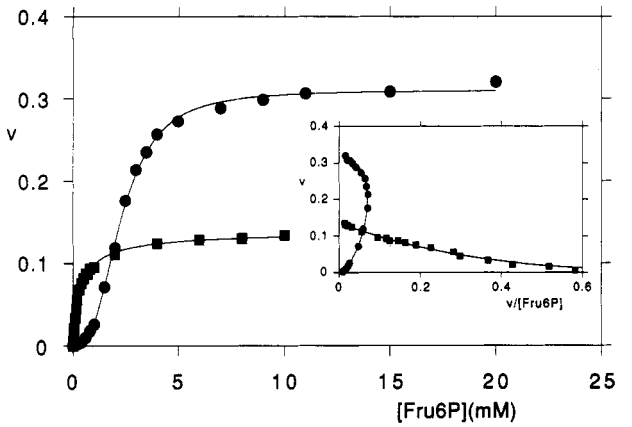

FIGURE 3: Dependence of rate on [Fru6P] for mutant DS129 in the absence of effectors (●) and in the presence of 1 mM GDP (■). Inset: Eadie–Hofstee plot, showing slight negative cooperativity with GDP, positive cooperativity without.

surprisingly by a smaller amount (for DA103, V_{max} is reduced by a factor of 30 in the forward reaction, by 12 in the back-reaction). Otherwise, DA103 behaves much like the wild-type enzyme, except that addition of GDP decreases the V_{max} and increases the $K_{m[ATP]}$ for DA103 (Figure 5c). This is characteristic of "mixed" inhibition (Palmer, 1985) and suggests that, in addition to its activating effect, GDP can also act as a competitive inhibitor for ATP.

Mutations in Asp127: DE127 and DY127. Asp127 is known to play a major role in catalysis from the enormous reduction in k_{cat} caused by mutation to serine (Hellinga & Evans, 1987); it probably acts as a base catalyst, abstracting the proton from the attacking 1-OH group of Fru6P. The two mutations DE127 and DY127 were constructed in an attempt to block the active site with bulky side chains. Both mutants have much reduced reaction rate (V_{max} reduced by factors of 120 for DE127 and 960 for DY127), but are more active than the Asp→Ser127 mutant (V_{max} reduced by a factor of 15 000–18 000). With respect to [Fru6P], DE127 shows a similar pattern to wild-type enzyme (Figure 4a), but with a greatly increased $S_{1/2}$ (by a factor of 160 without GDP, and by a factor of 320 with GDP). As in the wild-type enzyme, GDP converts the sigmoidal kinetics to hyperbolic. Thus it seems that the principal effect of this mutant is to decrease the Fru6P binding affinity (as well as the catalysis) while leaving the qualitative properties of the enzyme unchanged. In contrast, replacement of the same residue with Tyr (DY127) completely abolishes the cooperativity in the absence of GDP (Figure 4b). The $S_{1/2}$ is increased over that of the wild-type enzyme by a factor of 8. Surprisingly, in the presence of GDP, the $K_{m[Fru6P]}$ is very similar to that of the wild-type enzyme, so the bulky tyrosine side chain must point out of the active site to avoid obstruction. In addition, the enzyme is also inhibited by PEP, primarily by reducing V_{max}. Thus this mutation has effectively uncoupled homotropic from heterotropic allostery.

ES222. Finally, we note that the kinetic properties of ES222 are almost unchanged from that of the wild-type enzyme. Glu222 hydrogen bonds to the O_4 of the fructose moiety of Fru6P (Figure 1). As with other active site residues, it is relatively disordered in the unliganded state. The small size of the change caused by this mutation is thus surprising. It may be that the gap left by the mutation is large enough to accommodate a water molecule which could form a hydrogen bond with the fructose and thus compensate for the loss of the carboxyl group of Glu222.

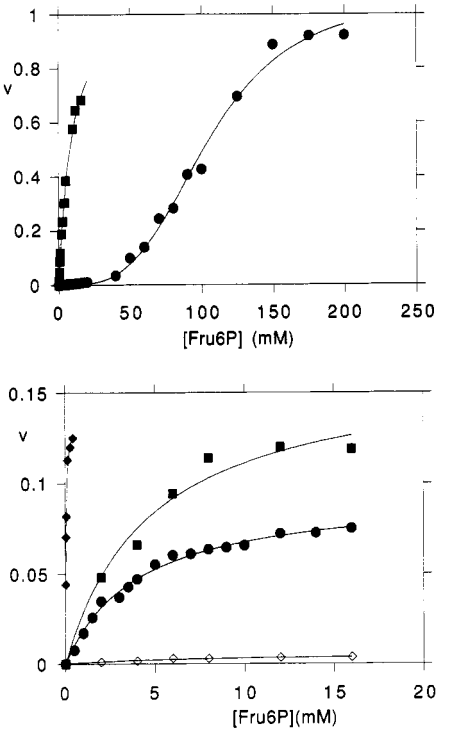

FIGURE 4: Dependence of rate on [Fru6P] for (a) DE127 [(●) no effectors; (■) +1 mM GDP] and (b) DY127 [(●) no effectors; (■) +0.5 mM GDP; (♦) +1 mM GDP; (◇) +2 mM PEP].

DISCUSSION

Perhaps the most informative data from this study are the effect of these mutations on catalytic rate. In phosphoryl-transfer reactions, enzymes can provide base and acid catalysts, positive charges for stabilizing negative charges on the phosphates, groups for positioning the donor and acceptor substrates and stabilizing the pentacoordinate transition state, and possibly mechanisms for preventing hydrolysis of the phosphoryl donor. The active site of PFK seems to contain most, if not all, of these functions. All the residues immediately around the catalytic site (Arg72, Asp103, Thr125, Asp127, Asp129, and Arg171) have been shown to be important, supporting the model represented in Figure 1 for direct phosphoryl transfer. Asp127 almost certainly acts as a base catalyst and is essential for efficient catalysis. Thr125, Arg72, and Arg171 probably stabilize the transition state, along with the peptide NH of Gly11; of these, Thr125 is the most critical, particularly for the back-reaction. Asp103 and particularly Asp129 are required to position the Mg^{2+} ion, which in turn stabilizes the transition state. The only missing ingredient is the essential monovalent cation (NH_4^+ or K^+), which has not been located by crystallography.

The mutants have been characterized using measurement of steady-state kinetics, which does not provide a direct measure of binding constants, so caution is needed in interpreting apparent changes in binding caused by mutation, particularly since in this allosteric enzyme the binding of different ligands is closely linked. Nevertheless, at least some of the measured parameters are consistent with the positions of mutated residues, though other changes seem surprising. None of these mutations makes a large change to the apparent affinity for the nucleotide substrates ATP in the forward reaction and ADP in the back-reaction, as monitored by K_m. Mutations on the Mg^{2+} binding side chains Asp103 and Asp129 impair the apparent binding of ADP, but surprisingly not to ATP. These carboxyl groups seem to be important primarily to stabilize the transition state over the substrate complexes.

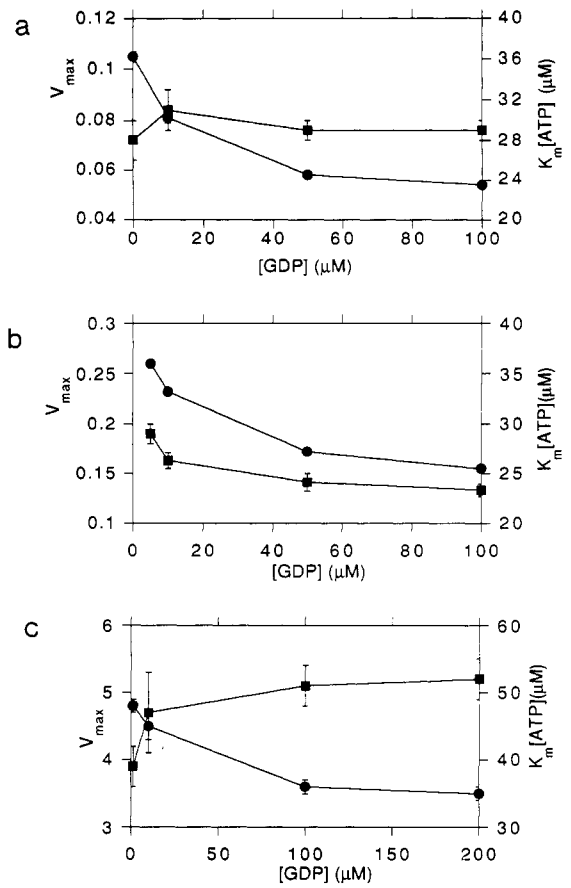

FIGURE 5: Effect of GDP on the ATP kinetic parameters of mutants TA125, DS129, and DA103. V_{max} (●) and $K_{m[ATP]}$ (■) values were obtained by fitting the data to the Michaelis–Menten equation. Error bars represent standard errors. (a) The kinetics with respect to ATP for mutant TA125 were measured in the presence of 2 mM Fru6P and varying concentrations of GDP. (b) The kinetics with respect to ATP for mutant DS129 were measured in the presence of 10 mM Fru6P and varying concentrations of GDP. (c) The kinetics with respect to ATP for mutant DA103 were measured in the presence of 1 mM Fru6P and varying concentrations of GDP.

Alterations in the affinity for Fru6P and Fru1,6P_2 are harder to interpret. Much of the binding energy for these molecules comes from interactions with the 6-phosphate group, some way from the catalytic site, and it is known that mutations in Arg162 and Arg243 severely impair Fru6P binding (Berger & Evans, 1990). The mutation ES222 was intended to reduce affinity for Fru6P by removing a charged hydrogen bond (Figure 1), but this mutant has almost identical properties to those of wild type. The two mutations of Asp127 were more successful in the aim of blocking the active site, particularly DE127, which shows a similar kinetic pattern to that for wild type, but with reduced apparent affinity for Fru6P. The highly cooperative kinetics of this enzyme suggest that, in spite of the large decrease in binding affinity for Fru6P, the equilibrium between the T and R states in this enzyme is essentially unchanged.

The dependence of reaction rate on the concentration of Fru6P is not fully understood for wild-type enzyme, which shows a highly sigmoidal response, even though binding of Fru6P in the absence of the second substrate ATP is hyperbolic (Berger & Evans, 1991; Deville-Bonne & Garel, 1992). The kinetic dependence represents the behavior of the enzyme in the presence of saturating ATP, and it is likely that the difference between the kinetic and binding data arises from antagonism between the two substrates, most likely with the ATP acting as an allosteric inhibitor, favoring a T-state conformation with low affinity for Fru6P. It is not clear from the structure how ATP might induce a transition to a low-affinity state, nor is it known whether this conformation is the same as that induced by the allosteric inhibitor PEP. However, the mutations TA125 and DY127 abolish the sigmoidal kinetics, while the DA103 and DS129 mutations preserve much of the cooperativity (DS129 has a reduced Hill constant). This suggests that the interactions with the γ-phosphate of ATP are crucial for cooperativity, supported also by the reduced Hill constant for the mutant RS72 (Berger & Evans, 1990).

One of the simplifications of the Monod model for allostery is the assumption that the allosteric effectors merely shift the equilibrium between R and T states, without changing their properties. A number of observations show that this cannot be strictly true for *E. coli* PFK. For example, at high concentrations of Fru6P, the wild-type enzyme is still activated by GDP, by an amount which depends on pH (Deville-Bonne et al., 1991a; Table I). In contrast to the wild-type enzyme, mutants DA103, TA125, and DS129 show a reduced V_{max} in the presence of GDP; i.e., GDP inhibits instead of activating the R-state enzyme. Binding of GDP to the effector site, remote from the active site, thus has two distinct effects: it stabilizes the high-affinity R state at the expense of the T state, leading to a reduction of cooperativity; and it modulates k_{cat} and probably K_m of the R state, perhaps by the subtle differences in the active site region seen by comparing the structures of the activated and the unliganded enzyme (Rypniewski & Evans, 1989). In DY127, GDP clearly modulates the apparent affinity for Fru6P as well as V_{max}; because this mutant is not cooperative, this effect is not obscured by sigmoidal kinetics, but the same may occur with wild type. In the DY127 mutant, high-affinity binding of Fru6P when GDP is present must require significant conformational change to accommodate the bulky tyrosine side chain outside the active site.

The inadequacies of the simple two-state concerted model are shown even more by the apparent negative cooperativity for TA125 and DS129 in the presence of GDP. The highly sigmoidal kinetics for the wild-type enzyme reflect the concerted transition from T to R of all sites in the tetramer. Negative cooperativity implies that binding of Fru6P at one site in the tetramer inhibits or prevents binding at the other sites, thus breaking the symmetry of the tetramer. However, these mutants do not show negative cooperativity in the reverse reaction, despite the activating effect of saturating ADP, which again points to the importance of interactions with the γ-phosphate of ATP.

REFERENCES

Banas, T., Gontero, B., Drews, V. L., Johnson, S. L., Marcus, F., & Kemp, R. G. (1988) *Biochim. Biophys. Acta 957*, 178–184.

Berger, S. A., & Evans, P. R. (1990) *Nature 343*, 575–576.

Berger, S. A., & Evans, P. R. (1991) *Biochemistry 30*, 8477–8480.

Blangy, D., Buc, H., & Monod, J. (1968) *J. Mol. Biol. 31*, 13–35.

Dente, L., Cesareni, G., & Cortese, R. (1983) *Nucleic Acids Res. 11*, 1645–1655.

Deville-Bonne, D., & Garel, J.-R. (1992) *Biochemistry 31*, 1695–1700.

Deville-Bonne, D., Bourgain, F., & Garel, J.-R. (1991a) *Biochemistry 30*, 5750–5754.

Deville-Bonne, D., Laine, R., & Garel, J.-R. (1991b) *FEBS Lett. 290*, 173–176.

Evans, P. R., & Hudson, P. J. (1979) *Nature 279*, 500–504.

Evans, P. R., Farrants, G. W., & Hudson, P. J. (1981) *Philos. Trans. R. Soc., Ser. B 293*, 53–62.

Hanahan, D. (1983) *J. Mol. Biol. 166*, 557–580.

Hellinga, H. W., & Evans, P. R. (1987) *Nature 327*, 437–439.

Jarvest, R. L., Lowe, G., & Potter, B. V. L. (1981) *Biochem. J. 199*, 427–432.

Kotlarz, D., & Buc, H. (1977) *Biochim. Biophys. Acta 484*, 35–48.

Palmer, T. (1985) *Understanding Enzymes*, 2nd ed., Ellis Horwood, Chichester.

Rypniewski, W. R., & Evans, P. R (1989) *J. Mol. Biol. 207*, 805–821.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.

Schirmer, T., & Evans, P. R. (1990) *Nature 343*, 140–145.

Shirakihara, Y., & Evans, P. R. (1988) *J. Mol. Biol. 204*, 973–994.

Uyeda, K. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 48*, 194–243.

Zoller, M. J., & Smith, M. (1983) *Methods Enzymol. 100*, 468–500.