Identification of Substrate Contact Residues Important for the Allosteric Regulation of Phosphofructokinase from *Eschericia coli*†

Aron W. Fenton, N. Monique Parichartttanakul, and Gregory D. Reinhart*

Department of Biochemistry and Biophysics and Center for Advanced Biomolecular Research, Texas A&M University, 2128 TAMU, College Station, Texas 77843-2128

Received February 18, 2003; Revised Manuscript Received March 27, 2003

ABSTRACT: The side chains of *Escherichia coli* phosphofructokinase (EcPFK) that interact with bound substrate, fructose 6-phosphate (Fru-6-P), are examined for their potential roles in allosteric regulation. Mutations that severely decrease Fru-6-P affinity and/or k_{cat}/K_m were created at each contact residue, with the exception of the catalytic base, D127. Even though Fru-6-P affinity was greatly decreased for R162E, M169A, E222A/H223A, and R243E, the mutated proteins retained the ability to be activated by MgADP and inhibited by phosphoenolpyruvate (PEP). R252E did not show an allosteric response to either MgADP or PEP. The H249E mutation retained MgADP activation but did not respond to PEP. R72E, T125A, and R171E maintained allosteric inhibition by PEP. Both R72E and T125A displayed a MgADP-dependent decrease in k_{cat} but no MgADP-dependent K-type effects. R171E maintained MgADP-dependent K-type activation but also displayed a MgADP-dependent decrease in k_{cat}. Localization of mutations that alter MgADP activation near the transferred phosphate group indicates the importance of the 1-methoxy region of Fru-6-P in allosteric regulation by MgADP. A region near the 6′-phosphate may be similarly important for PEP inhibition. R252 is uniquely positioned between the 1′- and 6′-phosphates of bound Fru-1,6-BP, and the mutation at this position may alter both allosterically responsive regions. The differential functions of specific regions in the Fru-6-P contact residues support different mechanisms for allosteric activation and inhibition. In addition, the lack of correlation between mutations that decrease Fru-6-P affinity and those that abolish allosteric communications supports the independence of affinity and allosteric coupling.

Escherichia coli phosphofructokinase 1 (EcPFK)[1] is a homotetramer which catalyzes the transfer of phosphate from MgATP to fructose 6-phosphate (Fru-6-P) producing MgADP and fructose 1,6-bisphosphate (Fru-1,6-BP) (*1*, *2*). Each subunit has a molecular mass of 34 kDa. The four active sites are formed at a dimer−dimer interface. In addition to exhibiting positive, homotropic cooperativity, binding of Fru-6-P is allosterically inhibited by phosphoenolpyruvate (PEP) and activated by MgADP (*3*). These two allosteric regulators compete for binding at the same allosteric binding site (*1*, *2*).

In X-ray crystallographic studies, the 6′-phosphate of Fru-1,6-BP appears to interact with R162, R243, and H249 of EcPFK (Figure 1) (*4*). In addition, the fructose moiety interacts with D127, R252, and E222. M169 provides the only hydrophobic contact with the substrate (*1*). Moreover, T125, R72, and possibly R171 interact with the 1′-phosphate of the product Fru-1,6-BP.

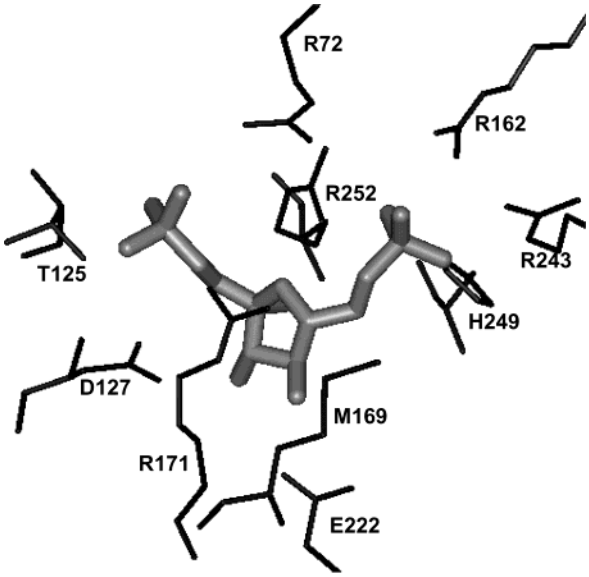

FIGURE 1: The active site as determined by Shirakihara and Evans (*1*). The product Fru-1,6-BP was cocrystallized with EcPFK. The Fru-1,6-BP molecule is in gray. Interacting residues are as indicated.

A large number of mutational studies have attempted to assign the role of each residue that contacts Fru-6-P with a function related to either binding, catalysis, or allosterism (*5*−*14*). The common experimental design to determine

† This work was supported by National Institutes of Health Grant GM33216 and Robert A. Welch Grant A-1543.

* To whom correspondence should be addressed. E-mail: gdr@tamu.edu.

[1] Abbreviations: DTT, dithiothreitol; EPPS, *N*-(2-hydroxyethyl)-piperazine-*N*′-3-propanesulfonic acid; Fru-1,6-BP, fructose 1,6-bisphosphate; PEP, phosphoenolpyruvate; PFK, phosphofructokinase; EcPFK, phosphofructokinase 1 from *Escherichia coli*; BsPFK, phosphofructokinase 1 from *Bacillus stearothermophilus*.

10.1021/bi034273t CCC: $25.00
Published on Web 05/08/2003

effects on allostery has been to assay in the presence and absence of one concentration of allosteric effector. This approach neglects to consider that any mutation may independently affect the binding affinity for the substrate, the effector, or the coupling between substrate and effector. The results of past mutagenesis studies have mainly been discussed in relationship to a structural model of the allosteric regulation of PFK from *Bacillus stearothermophilus* (BsPFK). This model was proposed on the basis of comparisons between solved crystal structures with either Fru-6-P and MgADP bound or with phosphoglycolate (a PEP analogue) bound. The structural model indicated that E161 and R162 were the key active site residues that were primarily responsible for the allosteric perturbation of Fru-6-P binding (*15*). However, upon further examination, E161 and R162 have been shown to play at most minor roles in the allostery exhibited by BsPFK (*16*). Therefore, there is currently a void in understanding which of the Fru-6-P contact residues are important for allosteric regulation.

The purpose of the current work is to reexamine residues that have been identified as Fru-6-P contacts for their potential role in allosteric regulation of EcPFK. Mutations have been made at each residue which has a side chain identified to be in contact with Fru-6-P, with the exception of the catalytic base D127 (*4*, *5*, *8*). The choices of amino acid replacements reflect substitutions reported in the literature and were chosen to decrease Fru-6-P affinity maximally. Recognizing the possible independent natures of substrate and allosteric effector binding affinities and the coupling between these ligands, Fru-6-P titrations of EcPFK were performed over a range of allosteric effector concentrations. Furthermore, since MgADP activation and PEP inhibition may function via different molecular mechanisms, Fru-6-P titrations were performed in varying concentrations of each effector. For most of the mutant proteins, saturation with Fru-6-P was not observed at all concentrations of allosteric effectors. However, trends due to allosteric effectors are evident.

MATERIALS AND METHODS

Materials. All chemical reagents were of analytical grade, purchased from Fisher or Sigma. Amicon Corp. Matrex Gel Blue A−agarose resin was used in protein purification. Creatine phosphate and the sodium salts of Fru-6-P and ADP were purchased from Sigma. Creatine kinase, aldolase, triosephosphate isomerase, and glycerol-3-phosphate dehydrogenase were obtained from Boehringer Mannheim. The sodium salt of ATP was obtained from either Boehringer Mannheim or Sigma.

Mutagenesis. pGDR16 (*17*), containing the EcPFK in pAlter I (Promega), was used with the altered sites II in vitro mutagenesis system (Promega) to construct mutations according to manufacturer's instructions. Mutagenesis primers were synthesized by the Gene Technologies Laboratory of the Institute of Developmental and Molecular Biology at Texas A&M University and are as follows: R72E, 5′-CGCGGAATTCCGGGAATTCCGCAGAACCGAGGAACG-3′; T125A, 5′-TGTCGATAGCGCCCGGC-3′; R162E, 5′-CATCACTTCCACCACGGAAATTTCCTGGTGAGAAGAAGAGGTGTCAC-3′; M169A, 5′-ACGGCCCGCCACTTCCACC-3′; R171E, 5′-CGTCAGATCTCCACAATATTCGCCCATCACTTCCACCACGG-3′; E222A/H223A, 5′-CATCACACATAGCTGCGGTAATCGCC-3′; R243E, 5′-GATGTGGCCCAGCACAGTTGCTTCGGTTTCACGACCGGTTTCTTTCT-3′; H249E, 5′-CCACCGCGCTGGATTTCGCCCAGCACAGTTGCGC-3′; R252E, 5′-GGCACCGGAGAACCACCTTCCTGGATGTGGCCCAGC-3′. Plasmid DNA was isolated using the Wizard SV kit (Promega). The DNA was sequenced across the site to confirm the presence of the desired mutation. Plasmids containing wild-type and mutant EcPFK genes were separately transformed into DF1020 cells for protein expression (*18*, *19*).

Protein Purification. Wild-type and mutant EcPFK proteins were purified from DF1020 cells expressing the desired plasmid construct using a modified method of Kotlarz and Buc (*20*) as described by Johnson et al. (*17*) except that all steps were performed at 4 °C. Protein concentration was determined by reading the absorbance in the absence of nucleotides using $\epsilon_{278} = 0.6$ cm^2 mg^{-1} (*21*). For mutations with very low activity (i.e., R252E), special care was required to ensure that hybrid tetramers, formed in situ between chromosomal wild type and plasmid mutant, were removed. Low-level expression of the chromosomal wild-type enzyme was present since DF1020 has the promoter region of *pfk*A removed but does not have the entire gene deleted (M. R. Lovingshimer, personal communication).

Kinetic Assays. Activity measurements were carried out in 1 mL of an EPPS buffer containing 50 mM EPPS−KOH (pH 8.0 at 8.5 °C), 10 mM NH_4Cl, 10 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 0.2 mM NADH, 250 μg of aldolase, 50 μg of glycerol-3-phosphate dehydrogenase, 5 μg of triosephosphate isomerase, and 3 mM ATP. Fru-6-P and either MgADP or PEP concentrations were varied as indicated. To prevent competition between MgADP and MgATP in the active site (*22*), MgADP was added as a solution containing equal molar MgADP and MgATP. In the absence of MgADP, 4 mM creatine phosphate and 40 μg/mL creatine kinase were added to convert MgADP contamination from substrate stocks to MgATP (*3*). To overcome triosephosphate isomerase inhibition by PEP, reactions with PEP present were supplemented with 50 μg of triosephosphate isomerase. EcPFK was added to initiate the enzymatic reaction, which was monitored at 340 nm over time. A unit of activity is defined as the amount of enzyme required to produce 1 μmol of Fru-1,6-BP per minute. MgADP contamination in MgATP was quantified using the method of Jaworek et al. (*23*). In the absence of the allosteric activator, MgADP, M169A and R243E displayed activation over the course of the assay. This may be evidence for subunit aggregation as previously noted in other systems (*24*). Rates were read from the linear portion of absorbance vs time plots. Rates vs substrate concentration were fit to the Hill equation (*25*):

$$\frac{v}{E_T} = \frac{k_{cat}[\text{Fru-6-P}]^{n_H}}{(K_{0.5})^{n_H} + [\text{Fru-6-P}]^{n_H}} \quad (1)$$

where v = initial rate, E_T = total enzyme active site concentration, k_{cat} = turnover number, $K_{0.5}$ = the concentration of Fru-6-P that yields a rate equal to one-half the maximal specific activity, and n_H = the Hill coefficient. For

Table 1: Kinetic Parameters in 3 mM ATP and at 8.5 °C

enzyme	k_{cat} (units/mg)	$K_{0.5}$ (mM)	k_{cat}/K [units/(mg·mM)][b]
wild type	61 ± 1	0.30 ± 0.01	205 ± 7
R171E	3.47 ± 0.01	0.86 ± 0.01	4.1 ± 0.1
R72E	0.303 ± 0.002	0.105 ± 0.002	2.9 ± 0.1
T125A	0.0146 ± 0.0003	0.58 ± 0.05	0.025 ± 0.002
R162E	ND[a]	ND	0.24 ± 0.01
E222A/H223A	ND	ND	0.23 ± 0.04
R243E	ND	ND	0.16 ± 0.01
M169A	ND	ND	$2.0 \pm 0.2 \times 10^{-4}$ [c]
H249E[c]	ND	ND	$1.5 \pm 0.1 \times 10^{-4}$
R252E	ND	ND	$6.5 \pm 0.4 \times 10^{-5}$

[a] Not determined due to lack of Fru-6-P saturation. [b] Determined directly as k_{cat}/K_m when data were fit to eq 2 or by dividing k_{cat} by $K_{0.5}$ when data were fit to eq 1. [c] Varied between preparations.

curves that show no evidence of saturation, the linear region of the Fru-6-P saturation curves were fit to

$$\frac{v}{E_T} = \left(\frac{k_{cat}}{K_m}\right)[\text{Fru-6-P}] \quad (2)$$

using the nonlinear least-squares fitting analysis of Kaleidagraph (Synergy) software.

Fluorescence Titration. Intensity of EcPFK fluorescence, at 0.36 μM subunit concentration, was measured in a 1 cm^2 cuvette with an SLM Model 4800 fluorometer equipped with updated electronics by ISS, Inc. (Champaign, IL), and thermostated at 8.5 °C. Excitation was at 300 nm, and emission was collected through a 1 mm thick Schott WG-335 filter. Titration experiments were performed in 50 mM EPPS−KOH (pH 8.0), 10 mM $MgCl_2$, 10 mM NH_4Cl, and 0.1 mM EDTA, and blank readings of buffer alone were subtracted from measured values.

RESULTS

The X-ray crystallographic protein−ligand structure of EcPFK with the product, Fru-1,6-BP, has been solved (*1*). Using this structure, Evans suggested that Fru-6-P makes contact with the side chains of residues D127, R162, M169, E222, R243, H249, and R252 (*4*). In addition, R72, T125, and possibly R171 interact with the transferred 1′-phosphate of Fru-1,6-BP. In the current study, mutations have been made at each of these positions, with the exception of the catalytic base D127 (*4*, *5*, *8*). Most of the amino acid replacements reflect previous mutations that have been reported in the literature (*5*−*14*). These modifications were expected to substantially decrease Fru-6-P affinity. Additionally, the mutation E222A was examined in concert with H223A to augment the effects of each alone.

Kinetic assays were performed at 8.5 °C in order to increase binding affinity for Fru-6-P (*26*). As shown in Table 1, each modification has a significant effect on PFK catalysis. The smallest perturbation is conferred by R171E, which diminishes k_{cat} more than 17-fold and the affinity for Fru-6-P about 3-fold. T125A and R72E diminish k_{cat} a further 1 and 2 orders of magnitude, respectively, while producing no greater effect on Fru-6-P binding. (R72E actually augments Fru-6-P binding nearly 3-fold.) All other mutations lead to even more extreme effects, with affinity diminished sufficiently so that saturation cannot be achieved with Fru-6-P concentrations up to 40 mM, and k_{cat}/K_m is diminished by at least 3 orders of magnitude for each variant. A comparison of Fru-6-P titrations of wild-type and mutant EcPFK proteins at varying concentrations of MgADP is shown in Figure 2. Fru-6-P titrations for the same set of mutations at varying concentrations of PEP are displayed in Figure 3. In both figures, arrows indicate increasing effector concentrations.

The effects of MgADP revealed in Figure 2 indicate that R162E, M169A, E222A/H223A, R243E, and H249E all retain the ability to be allosterically activated despite the poor ability of Fru-6-P to interact with the enzyme. R171E also exhibits MgADP activation of Fru-6-P binding although MgADP inhibits k_{cat} somewhat. In contrast, R72E and T125A do not exhibit K-type activation by MgADP up to 3.5 mM. MgADP binding is evident, however, because of a small, but reproducible, MgADP decrease in k_{cat}. To rule out the possibility that this inhibition, and that observed with R171E, is due to product inhibition by MgADP at the active site, MgATP concentration was raised in concert with the MgADP concentration (see Materials and Methods). In addition, the R252E protein seems to completely lack any allosteric response to MgADP (up to 3.5 mM).

Figure 3 demonstrates that only H249E and R252E lack PEP inhibition (up to 10 mM). All other mutated proteins display allosteric inhibition by PEP to some extent. Interestingly, both the 249 and 252 positions reside on the same 3_{10}-helix (*1*). The R252E mutation was unique in our mutant pool, in that it lacked both MgADP activation and PEP inhibition (Figures 2 and 3). No response to an effector can indicate that the effector is not binding to the mutant protein or that there is no longer coupling between the Fru-6-P binding site and the allosteric site. Therefore, we set about to determine if PEP can bind to the H249E protein and if both MgADP and PEP can bind to the R252E protein.

To test if PEP binds to the H249E mutant protein, the ability of PEP to compete with MgADP binding was tested. Figure 4 demonstrates that, at 60 mM Fru-6-P and 1.8 mM MgADP, PEP decreases activity in a concentration-dependent manner. Since Fru-6-P affinity of this mutant protein is insensitive to PEP, the only explanation to describe the decreased activity observed with increasing PEP is competition of PEP with MgADP in the allosteric site. Therefore, we conclude that PEP binds to the allosteric site of the H249E mutated protein.

PEP binding to R252E was monitored by changes in intrinsic trypophan fluorescence as shown in Figure 5. These data indicate that PEP binds to the protein in the absence of Fru-6-P in a manner similar to that of wild-type EcPFK (*27*). This binding is accompanied by a 6% increase in intensity. Addition of 3.5 mM MgADP causes a 14% decrease in fluorescence intensity. The presence of 3.5 mM MgADP during a PEP titration decreases the apparent PEP affinity from 8 to 66 μM, indicating competitive binding between MgADP and PEP to the allosteric site. Although the presence of 3 mM MgATP altered the magnitude of the intensity change observed with the PEP titrations, the same trends were observed for both PEP binding and PEP binding in the presence of 3.5 mM MgADP (data not shown). These results indicate that the R252E mutant protein binds both PEP and MgADP in the allosteric site with a K_d substantially below

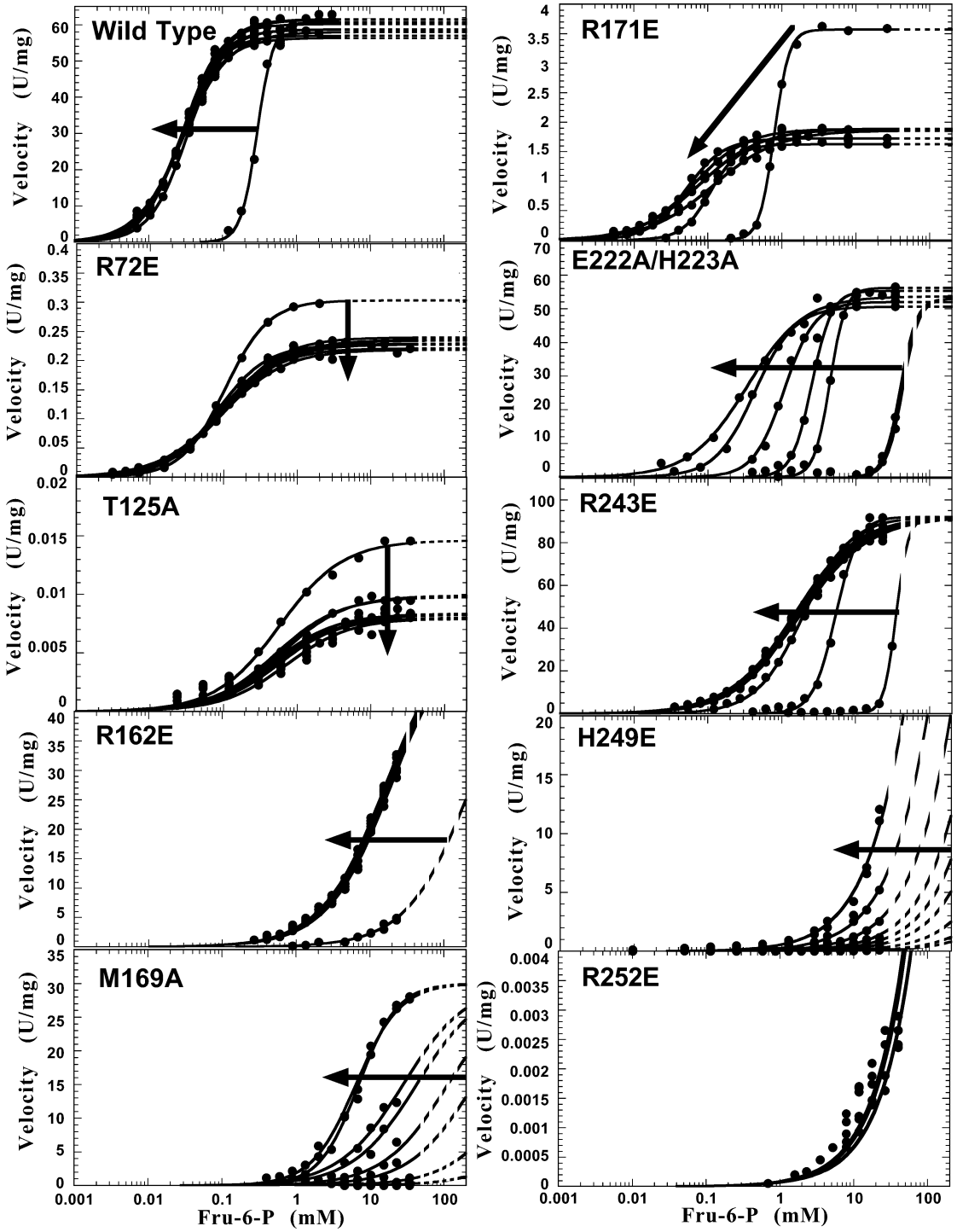

FIGURE 2: Fru-6-P titrations of wild-type and mutant EcPFK in the presence of varying concentrations of MgADP. Arrows indicate increasing MgADP concentrations from 0 to a maximum of 3.5 mM except for H249E and E222A/H223A, which were increased to 2.0 and 1.75 mM, respectively. Solid lines represent the best fit to eq 1 or 2. Dashed lines extend the best-fit predictions beyond the data. The minimum MgADP concentration greater than 0 was established by the ADP contamination in ATP. Creatine kinase and creatine phosphate were added to assays at 0 MgADP. Note the different scales used on the *Y*-axis.

the concentration used in the experiments presented in Figures 2 and 3.

DISCUSSION

In BsPFK, the role of E161 and R162 in allosteric regulation is at most minor, which does not support the previously proposed structural mechanism of allostery in this enzyme (*16*). Therefore, there is currently a void in the understanding of the molecular mechanism of allosteric communication of PFK from prokaryotes. The current study reexamines the possible roles that Fru-6-P contact residues may play in conveying allostery in EcPFK.

By design, most of the mutants examined in this report greatly decreased the affinity for Fru-6-P. However, both PEP and MgADP work by modifying the affinity displayed by wild-type EcPFK. The question was whether, after nearly abolishing the binding affinity for Fru-6-P, the actions of the allosteric ligands were also abolished. Quantification of

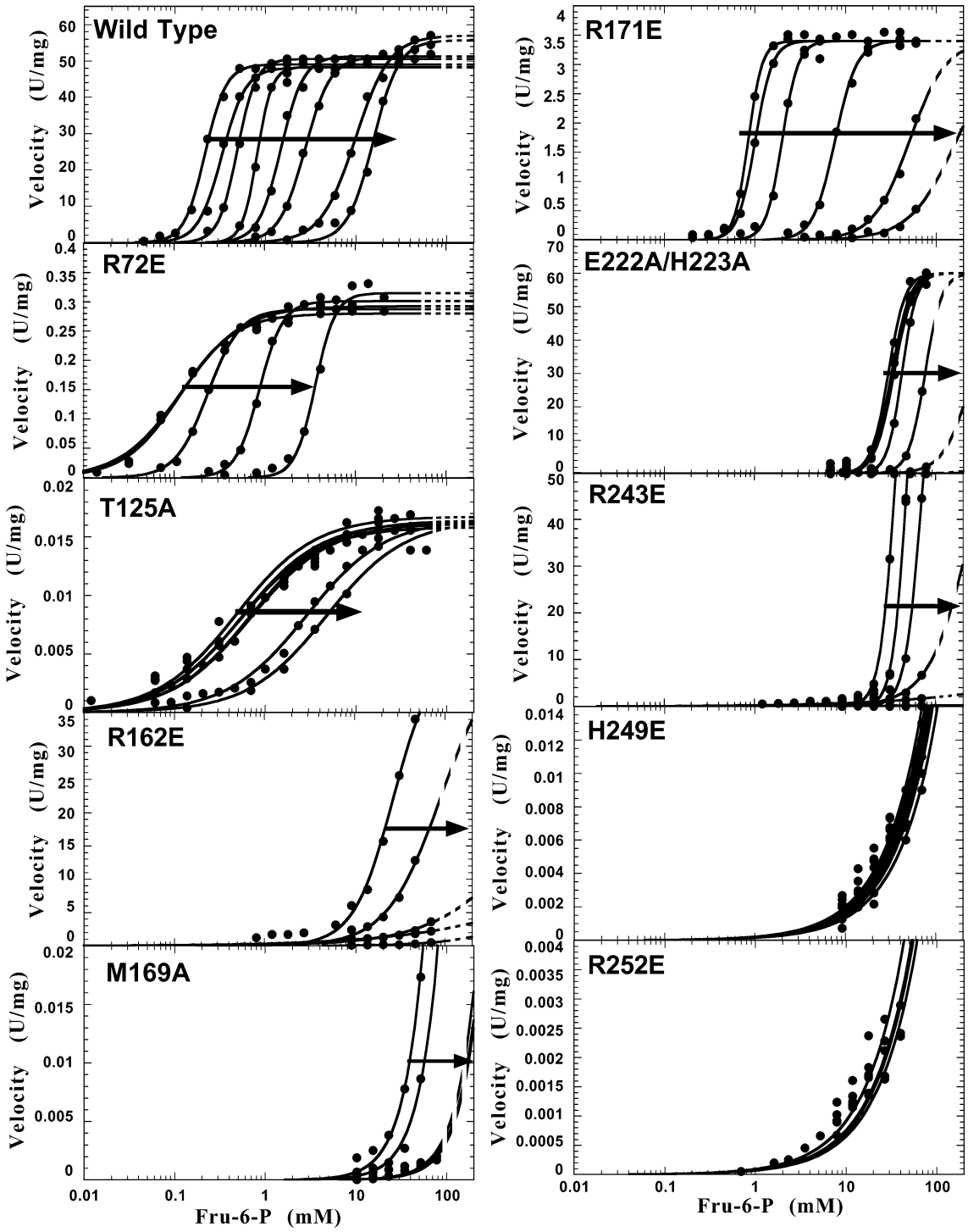

FIGURE 3: Fru-6-P titrations of wild-type and mutant EcPFK in the presence of varying concentrations of PEP. Arrows indicate increasing PEP concentrations. Minimum PEP concentrations are 0 in each case. Maximum concentrations of PEP equal 0.078 mM for R162E, 2.5 mM for R243E, 5.0 mM for R72E, M169A, R171E, E222A/H223A, and wild type, and 10 mM for the remaining variants. Solid lines represent the best fit to eq 1 or 2. Dashed lines extend the best-fit predictions beyond the data. Note the different scales used on the *Y*-axis.

the allosteric coupling was limited by the inability to saturate many of the mutated Fru-6-P binding sites particularly in the absence of MgADP. Therefore, only a qualitative comparison is possible (Figures 2 and 3).

R72E and T125A are the only mutations in our data set that disrupt MgADP activation without abolishing PEP inhibition (Figures 2 and 3). As with any mutational study, observed effects may be caused by the removal of the original residue or by the insertion of the replacement residue and may be due to changes in noncovalent bond interaction and/or changes in protein conformation. In addition, one must consider that even if there is a single interaction largely responsible for transmitting allostery, mutations that alter the environment in which the interaction functions might also alter the allosteric signal. Therefore, a more compelling argument for defining the region of the Fru-6-P contact residues important for MgADP activation may be that severe mutations can be added to the other residues considered to be Fru-6-P contact residues without disruption of MgADP activation. Since both R72 and T125 interact with the

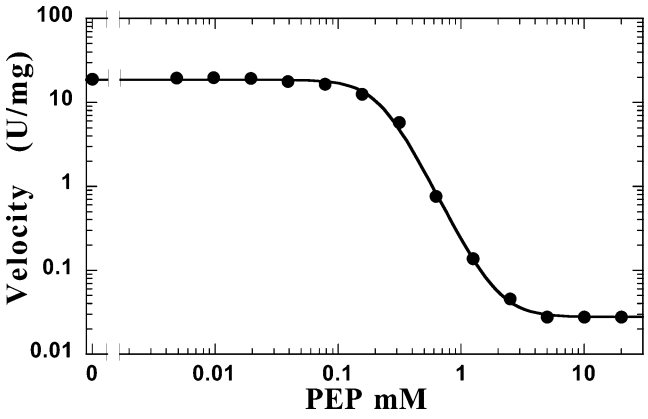

FIGURE 4: Competition between PEP and MgADP at the allosteric site of the H249E mutation. Activity was followed as a function of increasing PEP concentration and in the presence of 60 mM Fru-6-P and 1.8 mM MgADP.

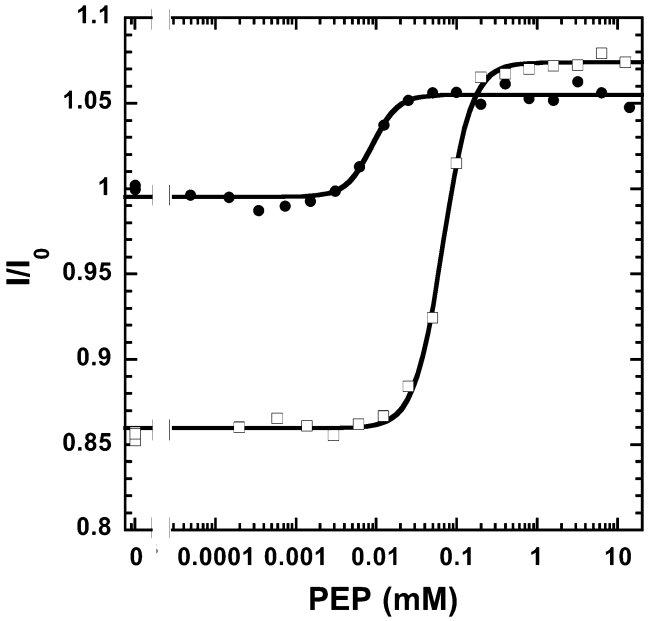

FIGURE 5: Change in fluorescent intensity of R252E due to PEP titrations. PEP titrations were in the presence (□) or absence (●) of 3.5 mM MgADP.

1′-phosphate of Fru-1,6-BP (*4*), MgADP binding in the allosteric site must elicit some change or changes near the transferred phosphate group in the active site. Given that MgADP promotes the binding of Fru-6-P in the absence of MgATP (*22*), a likely explanation is that it does so by improving the interactions near the 1-methoxy group.

Further support for this interpretation is offered by considering the effects on k_{cat}. R72E, T125A, and R171E all cause k_{cat} to become MgADP sensitive, and all are located near the 1′-phosphate of the Fru-1,6-BP product. The observed k_{cat} effects may be due to any number of alterations at the catalytic center. Independent of the cause, the observed effects support the idea that MgADP activation leads to perturbations in the phosphoryl-transfer region near carbon 1 of Fru-6-P.

R72E, T125A, and R171E, which cause k_{cat} to be MgADP sensitive, do not cause k_{cat} to be sensitive, nor Fru-6-P binding to be insensitive, to PEP (Figure 3). These observations suggest that residues important for PEP inhibition are more distant from the catalytic center. H249E is the only

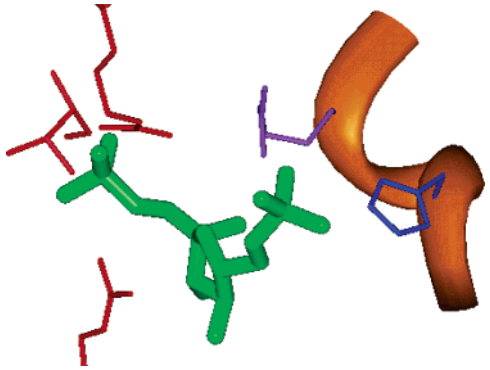

FIGURE 6: Localization of the effector-specific groups of Fru-6-P contact residues. The bond product is Fru-1,6-BP and is in green. Mutations at the positions of R72, T125, and R171 display altered allosteric responses to MgADP and are colored in red. H249E displays an altered allosteric response to PEP, and the residue position is colored blue. R252E is the only mutation that caused a complete loss of allosteric response. Therefore, the 252 position is colored purple to reflect involvement in both allosteric mechanisms. The backbone of the 3_{10}-helix containing the 249 and 252 positions is in an orange ribbon.

mutant that does not respond to PEP but maintains activation by MgADP. The lack of PEP inhibition by H249E is seen even though PEP still binds to the allosteric site (Figure 4). As with MgADP activation discussed above, the negative argument is the most convincing for defining what regions of the Fru-6-P contact residues are important in PEP inhibition. Severe mutations can be introduced at all positions, yet only H249E uniquely disrupts PEP inhibition without abolishing MgADP activation. H249 interacts with the 6′-phosphate of Fru-6-P (*4*), which suggests that an interaction (or interactions) near the 6′-phosphate is responsible for relaying PEP inhibition to Fru-6-P binding.

In our mutation set, R252E uniquely removes both MgADP activation and PEP inhibition. R252 is seen to interact with the 2′-hydroxyl of Fru-6-P in EcPFK structures (*4*). Consequently, the side chain of R252 is positioned between the 1′- and 6′-phosphates of the bound Fru-1,6-BP product. At this central location, R252E may alter the interactions and/or environment at the 1′-phosphate region, which are responsible for MgADP activation, as well as interactions and/or environment at the 6′-phosphate region, which are responsible for PEP inhibition.

Overinterpretation of these data sets should be avoided for several reasons. First, these data sets were collected at a MgATP concentration that is saturating for wild type but may not be saturating for any given mutant protein. Consequently, some of the effects of MgADP or PEP could possibly derive from modifying the relative degree of MgATP saturation, since MgATP is known to antagonize Fru-6-P binding (*28*). In addition, couplings between MgATP and PEP or MgADP have previously been determined to be small but nonzero in magnitude (*22*, *27*), giving rise to an indirect influence on Fru-6-P binding. Finally, since saturation by Fru-6-P is not reached, the Hill coefficient for Fru-6-P binding cannot be determined. We have previously demonstrated that the magnitude of apparent heterotropic coupling can be influenced by a change in the homotropic interactions present (*26*, *29*). Nonetheless, the qualitative trends evident in Figures 2 and 3 are substantial enough that even if MgATP or homotropic interactions are contributing, genuine heterotropic effects must be involved as well.

Overall, the present study identifies two regions that are important to allosteric regulation. The region near the 1′-phosphate group of the bound Fru-1,6-BP product appears to function in conveying MgADP activation of Fru-6-P binding. A region near the 6′-phosphate seems to play the same role in transmitting PEP inhibition. The R252 position appears to be centrally positioned so that the R252E mutation disrupts both MgADP activation and PEP inhibition. Furthermore, since mutations in different regions of the Fru-6-P contact residues differentially alter PEP and MgADP regulation, mechanisms for allosteric activation and inhibition likely proceed by different allosteric pathways.

There may be several reasons why these regions have not been identified in earlier studies. First, the dominating ideology of the classical two-state theories diminishes the expectation that allosteric effects can be independent of ligand affinity as predicted by linkage analysis (*30*−*32*). In the current study, the lack of correlation between mutations that greatly reduce Fru-6-P affinity and those that disrupt allosteric communication underscores the independence of these parameters. Therefore, characterizing allostery based on assaying substrate affinity in the absence of allosteric effector and comparing the same parameter determined in the presence of a single concentration of allosteric effector neglect the possibility that the decreased affinity of the allosteric effectors might have been caused by the mutation. Second, all of the Fru-6-P contact residues have not previously been probed in a single study, limiting the conclusions that could be drawn within a given work. In addition, the desire to have a single molecular mechanism that can easily explain both activation and inhibition has influenced the interpretation of many experiments. Recognizing the different allosteric roles played by the different regions of residues that contact the Fru-6-P substrate, as determined in the current study, will provide a framework for more in-depth studies into the various molecular mechanisms of allosteric communication in the future.

ACKNOWLEDGMENT

We thank Jason L. Johnson, Southwestern Oklahoma State University, for help in constructing some of the modified proteins.

REFERENCES

1. Shirakihara, Y., and Evans, P. R. (1988) *J. Mol. Biol. 204*, 973−994.
2. Ryniewski, W. R., and Evans, P. R. (1989) *J. Mol. Biol. 207*, 805−821.
3. Blangy, D., Buc, H., and Monod, J. (1968) *J. Biol. Chem. 31*, 13−35.
4. Evans, P. R. (1992) in *Proceedings of The Robert A. Welch Foundation: XXXVI. Regulation of Proteins by Ligands*, pp 39−54, The Robert A. Welch Foundation, Houston, TX.
5. Hellinga, H. W., and Evans, P. R. (1987) *Nature 327*, 437−439.
6. Berger, S. A., and Evans, P. R. (1990) *Nature 343*, 575−576.
7. Berger, S. A., and Evans, P. R. (1992) *Biochemistry 31*, 9237−9242.
8. Laine, R., Deville-Bonne, D., Auzat, I., and Garel, J.-R. (1992) *Eur. J. Biochem. 207*, 1109−1114.
9. Zheng, R.-L., and Kemp, R. G. (1992) *J. Biol. Chem. 267*, 23640−23645.
10. Auzat, I., Le Bras, G., and Garell, J.-R. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5242−5246.
11. Auzat, I., Byrnes, W. M., Garel, J.-R., and Chang, S. H. (1995) *Biochemistry 34*, 7062−7068.
12. Zheng, R.-L., and Kemp, R. G. (1994) *J. Biol. Chem. 269*, 18475−18479.
13. Zheng, R.-L., and Kemp, R. G. (1995) *Biochem. Biophys. Res. Commun. 3*, 765−770.
14. Wang, X., Deng, Z., and Kemp, R. G. (1998) *Biochem. Biophys. Res. Commun. 250*, 466−468.
15. Schirmer, T., and Evans, P. R. (1990) *Nature 343*, 140−145.
16. Kimmel, J. L., and Reinhart, G. D. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 3844−3849.
17. Johnson, J. L., Lasagna, M. D., and Reinhart, G. D., (2001) *Protein Sci. 10*, 2186−2194.
18. French, B. A., Valdez, B. C., Younathan, E. S., and Chang, S. H. (1987) *Gene 59*, 279−283.
19. Daldal, F. (1983) *J. Mol. Biol. 168*, 285−305.
20. Kotlarz, D., and Buc, H. (1982) *Methods Enzymol. 90*, 60−70.
21. Kotlarz, D., and Buc, H. (1977) *Biochim. Biophys. Acta 484*, 35−48.
22. Johnson, J. L, and Reinhart, G. D. (1994) *Biochemistry 33*, 2635−2643.
23. Jaworek, D., Gruber, W., and Bergmeyer, H. U. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) Vol. 4, pp 2127−2131, Academic Press, New York.
24. Reinhart, G. D., and Lardy, H. A., (1980) *Biochemistry 19*, 1491−1495.
25. Hill, A. V. (1910) *J. Physiol. 40*, 190−224.
26. Fenton, A. W., and Reinhart, G. D. (2002) *Biochemistry 41*, 13410−13416.
27. Johnson, J. L., and Reinhart, G. D. (1997) *Biochemistry 36*, 12814−12822.
28. Johnson, J. L, and Reinhart, G. D. (1992) *Biochemistry 31*, 11510−11518.
29. Reinhart, G. D. (1988) *Biophys. Chem. 30*, 159−172.
30. Reinhart, G. D. (1983) *Arch. Biochem. Biophys. 225*, 389−401.
31. Weber, G. (1972) *Biochemistry 11*, 864−878.
32. Weber, G. (1975) *Adv. Protein Chem. 29*, 1−83.

BI034273T