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ARTICLE *in* BIOCHEMISTRY · MAY 1995

Impact Factor: 3.02 · DOI: 10.1021/bi00021a018 · Source: PubMed

Role of Residue 161 in the Allosteric Transitions of Two Bacterial Phosphofructokinases†

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Received January 23, 1995; Revised Manuscript Received March 20, 1995®

ABSTRACT: The loop between α-helix 6 and β-strand F of the phosphofructokinase (PFK) from *Bacillus stearothermophilus* is proposed to be important in the allosteric transition of the enzyme [Schirmer, T., & Evans, P. R. (1990) *Nature 343*, 140−145]. Except for residue 161, the amino acids within the loop are similar between *B. stearothermophilus* PFK (BsPFK) and the PFK from *Escherichia coli* (EcPFK). In the former enzyme, residue 161 is a glutamate, while in the latter it is a glutamine. We have used site-directed mutagenesis to investigate the importance of residue 161 for the allosteric regulation of the two enzymes by phosphoenolpyruvate (PEP), an inhibitor, and GDP, an activator. In BsPFK, glutamate 161 has been changed to a glutamine and an alanine, while in EcPFK, glutamine 161 has been changed to a glutamate, an arginine, and an alanine. The kinetic parameters of the mutant enzymes were similar to those of the respective wild types, indicating that residue 161 is not directly involved in substrate binding and catalysis. One of the EcPFK mutants, Q161A, though activated normally by GDP, was completely insensitive to PEP. This indicates that the hydrogen-bonding ability of residue 161 is critical for PEP inhibition of EcPFK and suggests that GDP activation and PEP inhibition follow different structural pathways in EcPFK. The BsPFK mutant enzymes were less sensitive to PEP inhibition and more sensitive to GDP activation, suggesting that inhibition and activation are opposed and follow a common structural pathway in agreement with a concerted allosteric mechanism.

Phosphofructokinase (PFK,[1] EC 2.7.1.11) catalyzes the first committed step of glycolysis, the transfer of the γ-phosphate of ATP to fructose 6-phosphate (Fru-6P) to produce ADP and fructose 1,6-bisphosphate. A divalent Mg^{2+} ion, as well as a monovalent NH_4^+ or K^+ ion, is required for catalysis. In bacteria, PFK is allosterically inhibited by phosphoenolpyruvate (PEP) and activated by ADP (or GDP). Bacterial PFK is a tetramer of identical subunits each 36 000 Da in size, and exists as a dimer of rigid dimers. There are four active sites and four effector sites into which both PEP and ADP (or GDP) bind.

The PFKs from the two bacteria *Escherichia coli* and *Bacillus stearothermophilus* are remarkably similar in structure (Evans *et al.*, 1981; Shirakihara & Evans, 1988): their subunits have all the same secondary structural elements, their subunit α-carbon traces are nearly superimposable, and they share 55% amino acid identity. Nevertheless, the two enzymes have different regulatory properties. The saturation by the substrate Fru-6P of EcPFK is markedly cooperative (Blangy et al., 1968), while that of BsPFK is hyperbolic (Valdez et al., 1989), and their regulation by GDP is also somewhat different: GDP is a strong activator of EcPFK in the presence of saturating MgATP and nonsaturating Fru-6P concentrations but a weak activator of BsPFK under these conditions (Byrnes *et al.*, 1995).

The heterotropic regulation of EcPFK by PEP and GDP has been explained in terms of the concerted two-state model of Monod, Wyman, and Changeux [MWC model (Monod *et al.*, 1965)], in which the protein molecule exists in equilibrium between two states: a low-activity T state that binds PEP and a high-activity R state that binds GDP (Blangy *et al.*, 1968). Schirmer and Evans (1990) have uncovered the structural basis for the allosteric transition of BsPFK by comparing crystal structures of the enzyme obtained in the presence of inhibiting versus activating conditions. Upon this transition, the 6F loop, which is composed of residues Thr-156 to Arg-162, rearranges its structure (Figure 1). Arg-162, which points away from the active site in the T-state, swings back into the active site during the T-to-R transition, binding the phosphate group of Fru-6P in the R-state. At the same time, Glu-161, which in the T-state projects into the dimer−dimer interface where it forms a salt bridge with Arg-243 and interacts with active site residue Arg-252

† This work was supported by National Institutes of Health Grant DK31676 awarded to S.H.C. and by grants from the CNRS (ERS029), the University Paris 6 (9270300), and the BSN group.

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® Abstract published in *Advance ACS Abstracts,* May 15, 1995.

[1] Abbreviations: PFK, fructose-6-phosphate 1-kinase; EcPFK and BsPFK, the phosphofructokinases from the bacteria *Escherichia coli* and *Bacillus stearothermophilus*, respectively; *ecpfk* and *bspfk*, genes encoding phosphofructokinase in these two strains of bacteria, respectively; Bs E161Q and E161A, mutant *B. stearothermophilus* PFKs having a glutamine and an alanine, respectively, instead of a glutamate at position 161; Ec Q161E, Q161R, and Q161A, mutant *E. coli* PFKs having a glutamate, an arginine, and an alanine, respectively, instead of a glutamine at position 161; X-gal, 5-bromo-4-chloro-3-indoyl-β-D-galactoside; IPTG, isopropylthio-β,D-galactoside; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; YT, medium containing yeast extract and tryptone; 6F loop, region between α-helix 6 and β-strand F of BsPFK.

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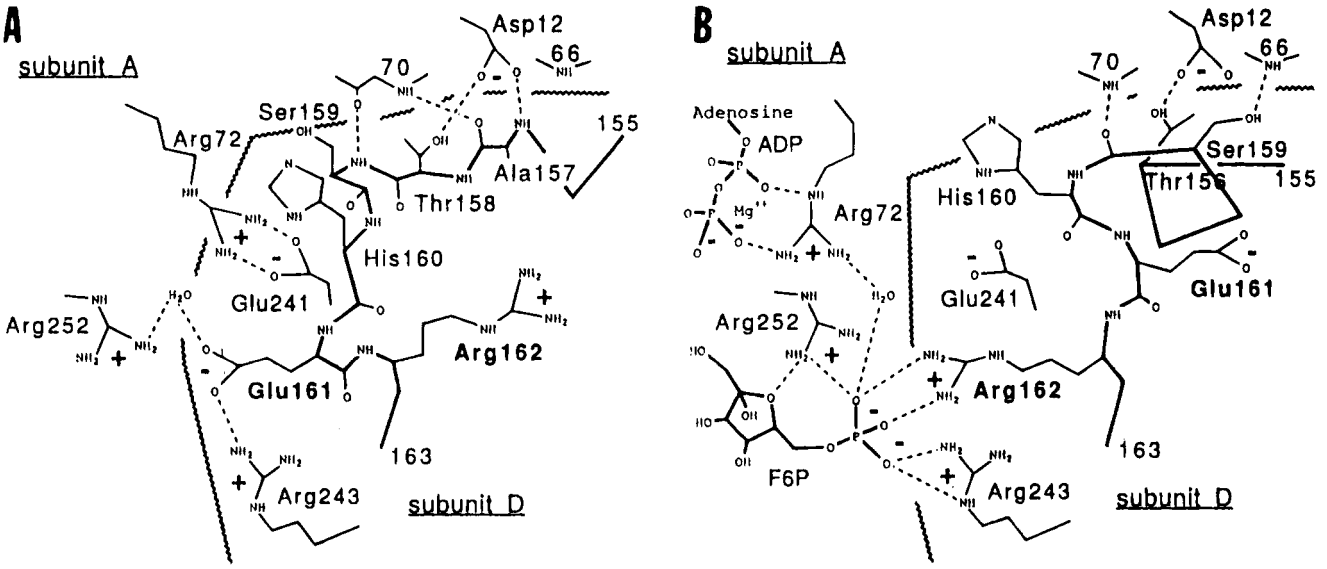

FIGURE 1: Reorganization of the 6F loop (156–162) of *B. stearothermophilus* PFK. The wavy line represents the boundary between dimers. (A) T-state structure, showing Glu-161 in the Fru-6P site. (B) R-state structure, showing the phosphate of Fru-6P bound by Arg-162 and Arg-243. Courtesy of Dr. P. R. Evans and permission from *Nature* [Schirmer, T., & Evans, P. R. (1990) *Nature 343*, 140–145]. Copyright 1990 Macmillan Magazines, Ltd.

	161
B. stearothermophilus	T A T S H **E** R
E161Q	T A T S H **Q** R
E161A	T A T S H **A** R
E. coli	T S S S H **Q** R
Q161E	T S S S H **E** R
Q161R	T S S S H **R** R
Q161A	T S S S H **A** R

FIGURE 2: Amino acid sequences of the 6F loops of *B. stearothermophilus* PFK, *E. coli* PFK, and the five mutant enzymes. Residue 161 is shown in enlarged, bold print.

through a water molecule, has rotated away in the R-state. The 6F loop and residues Arg-162 and Glu-161 within it are thus proposed to be important in the allosteric transition of BsPFK. Most of the residues within the 6F loop, including Arg-162, are conserved between BsPFK and EcPFK (Hellinga & Evans, 1985), but Glu-161 is not. In EcPFK, residue 161 is a glutamine (Figure 2).

EcPFK has not yet been successfully crystallized in the T-state, and assumptions about which residues participate in its allosteric T-to-R transition are based on structural similarity to BsPFK. X-ray diffraction studies suggest that the Glu-161 is important in stabilizing the T-state of BsPFK. Kinetic measurements of the inhibition of EcPFK by PEP indicate that the R-to-T transition can take place when residue 161 is a glutamine. In order to determine if residue 161 is really essential for the regulatory properties of PFK, and if any differences between BsPFK and EcPFK can be traced to the difference between Glu and Gln at position 161, we have constructed a set of mutants of both BsPFK and EcPFK with changes at this position. Glu-161 of BsPFK has been changed to glutamine and to alanine which lacks hydrogen bonding ability and is smaller. Gln-161 of EcPFK has been changed to glutamate and alanine, and also to arginine in order to have a positively charged residue at position 161. The abilities of PEP and GDP to inhibit and activate, respectively, the five mutant and the two wild-type enzymes were studied using steady-state kinetics. The results of these studies are presented.

MATERIALS AND METHODS

Enzymes, Chemicals, and Oligonucleotides. Restriction endonucleases, T4 DNA ligase, DNA polymerase (Klenow fragment), T4 polynucleotide kinase, and other enzymes used for cloning or *in vitro* mutagenesis were from New England Biolabs (Beverly, MA), Gibco-BRL (Grand Island, NY), or United States Biochemical (Cleveland, OH). X-gal and IPTG were from Gibco-BRL. The auxiliary enzymes aldolase, α-glycerophosphate dehydrogenase, and triosephosphate isomerase were from Sigma Chemical Co. (St. Louis, MO). Substrates ATP and Fru-6P, and effectors PEP and GDP, as well as the Cibacron Blue 3GA agarose (type 3000-CL-L), were also from Sigma. CNBr-activated Sepharose 4B and Blue Dextran 2000 were from Pharmacia (Uppsala, Sweden). The mutagenic oligonucleotides and the sequencing primers were synthesized on an Applied Biosystems 380A DNA Synthesizer. All oligonucleotides were purified in a two-step procedure involving (1) reverse-phase chromatography on an Oligo-Pak (Millipore, Milford, MA) purification column and (2) preparative 20% polyacrylamide gel electrophoresis. The purity and length of each oligonucleotide was verified by first 5′ end-labeling the primer using [γ-^{32}P]ATP and T4 polynucleotide kinase and then subjecting it to electrophoresis on an analytical 20% polyacrylamide gel and autoradiography.

Site-Directed Mutagenesis. The EcPFK gene was excised from a recombinant *ecpfk*/pUC18 plasmid (Hellinga & Evans, 1985) and subcloned into M13mp19 via its *Nar*I and *Kpn*I restriction sites. Similarly, the BsPFK gene contained within a 2.5 kilobase *Eco*RI/*Cla*I fragment that had been cloned into pBR322 (French & Chang, 1987) was subcloned into M13mp18 *via* its *Eco*RI and *Acc*I sites. The single-stranded DNA produced from either M13 vector contained the coding strand of the *pfk*.

Competent DH5αF′ *E. coli* cells (Gibco-BRL) were transformed with the recombinant M13 RF DNA and then plated by the top-agar method onto YT (yeast extract/tryptone) agar plates containing X-gal and IPTG, which allow blue/white color selection. After overnight incubation at 37 °C, a clear (white) plaque harboring the *ecpfk* or *bspfk* insert was picked and used to infect DH5αF′ cells. The infected cells were grown in liquid YT medium for 6 h at 37 °C. After centrifugation, the supernatant of the cell culture, which contained M13 phage particles, was used to infect fresh *E. coli* BW313 cells (*dut*$^-$, *ung*$^-$). The infected BW313 cells were grown overnight at 37 °C in YT medium containing uridine at 25 μg/mL. Uracil-containing single-stranded DNA was then prepared from the supernatant of the infected cell culture (Kunkel *et al.*, 1987) and used as a template for *in vitro* mutagenesis as described by Zoller and Smith (1983). The mutagenic oligonucleotides were designed to change codon 161 in *bspfk* from that for a glutamate (GAG) to that for either a glutamine (CAG) or an alanine (GCG). In *ecpfk*, the mutagenic oligos were designed to change codon 161 from that for a glutamine (CAG) to that for a glutamate (GAA), an arginine (CGT), or an alanine (GCT). After mutagenesis, competent DH5αF′ cells were transformed with the reaction mixture containing the mutated DNA and then plated onto YT agar plates. Transformants were screened for the mutation by (1) plaque hybridization using the mutagenic oligonucleotide labeled with ^{32}P as a probe (Seong & RajBhandary, 1987) and (2) partial sequencing of the single-stranded DNA from positives identified in the plaque hybridization. A single positive transformant was then selected, single-stranded DNA was prepared from it, and the entire coding region of the mutated gene was sequenced by the dideoxy method (Sanger, 1977) using a Sequenase kit (United States Biochemical) and a battery of intragenic primers to verify that no unintentional mutations had been introduced. This was done for all five mutated genes.

Expression and Purification of PFKs. The mutated BsPFK genes were excised from M13mp18 and inserted into the *Eco*RI/*Hin*dIII sites of the plasmid pUC18. Similarly, the mutated EcPFK genes were excised from M13mp19 and inserted into the *Hin*dIII site of pUC18. The recombinant pUC18 plasmids were then transformed into competent cells of a PFK-deficient *E. coli* strain (DF 1020) (Daldal, 1983) for expression. The transformed DF 1020 cells were grown to stationary phase in Luria Broth containing ampicillin (50 μg/mL), and the cells were pelleted. The procedures used to purify BsPFK and EcPFK were somewhat different. For BsPFK, the cells are resuspended in 50 mM Tris-HCl, pH 7.4, 1 mM DTT, 1 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride (buffer A) and sonicated. BsPFK and its mutants were purified from the crude extract in a two-step procedure that involved heat-treatment at 70 °C followed by affinity chromatography on a Cibacron Blue 3GA agarose column (Valdez *et al.*, 1989). The wild-type and two mutant BsPFK enzymes were eluted from the column with a 0.25−1.5 M NaCl gradient; all three eluted at approximately 1.1 M NaCl. Like wild-type BsPFK, the E161Q and E161A mutant enzymes were both stable when heated at 70 °C. Heat treatment was not used in purifying the wild-type and mutant EcPFK enzymes since they are not thermostable. Rather, the cells were disrupted with alumina beads in P buffer (20 mM phosphate, pH 7.6, 1 mM magnesium acetate, and 2 mM DTT). The crude extract obtained was loaded directly onto a column of Blue Dextran covalently coupled to Sepharose 4B and purified essentially as described (Deville-Bonne *et al.*, 1991; Auzat, 1993). The column was washed with 0.5 M NaCl in P buffer, and then the native or modified EcPFK was eluted with 2 mM ATP and 10 mM magnesium acetate in P buffer. For all enzymes, column fractions having the highest PFK activity were pooled. Each enzyme preparation was shown to be pure by the presence of a single band on a 12% SDS−polyacrylamide gel stained with Coomassie Brilliant Blue. Purified wild-type or mutant BsPFK enzymes were concentrated by dialysis against 50% glycerol in buffer A at 4 °C. Purified wild-type or mutant EcPFK enzymes, on the other hand, were concentrated by ultrafiltration in an Amicon cell (Beverly, MA). The enzymes concentrated in 50% glycerol were stored at −20 °C, and the enzymes concentrated by ultrafiltration were precipitated with 55% ammonium sulfate and then stored in the presence of 5 mM Fru-6P at 4 °C.

Activity Assays. PFK activity was measured at 30 °C using an assay system (Kotlarz & Buc, 1982) that coupled the production of fructose 1,6-bisphosphate to the oxidation of NADH. The assay solution contained 100 mM Tris-HCl, pH 8.2, 10 mM $MgCl_2$, 5 mM NH_4Cl, 0.20 mM NADH, and the coupling enzymes aldolase (20 μg/mL), triosephosphate isomerase (10 μg/mL), and α-glycerophosphate dehydrogenase (10 μg/mL). An ATP-regenerating system that utilizes creatine phosphate (1 mM) and creatine phosphokinase (10 μg/mL) was used in all EcPFK-catalyzed reactions to prevent activation by ADP produced in the reaction. When studying GDP activation, the regenerating system was not used. It was found to be unnecessary for BsPFK and its mutants since they are not activated to any significant degree by GDP except in the presence of PEP. For each assay, the change in absorbance at 340 nm was measured for at least 1 min using an Hitachi UV-2000 spectrophotometer. Reactions were initiated by addition of PFK. One initial velocity unit is defined as the number of micromoles of fructose 1,6-bisphosphate formed per minute; there are 12.4 initial velocity units per ΔA_{340}/min unit. In order to obtain kinetic parameters, initial velocity data were fit to either the Michaelis−Menten equation for hyperbolic kinetics or the Hill equation for sigmoidal kinetics. All curve-fitting was performed by nonlinear regression analysis using the program INPLOT (GraphPad, Inc.) or Kaleidagraph (Biosoft, Inc.). The errors presented in Tables 1−4 are standard errors obtained from curve-fitting.

RESULTS

Steady-State Kinetic Parameters. Substrate saturation of the wild-type and mutant enzymes was studied using steady-state kinetics. In agreement with previously published results, the two wild-type enzymes were found to have different Fru-6P saturation profiles in the presence of saturating MgATP concentration: whereas the curve for EcPFK was highly sigmoidal with a Hill number of 3.9 (Blangy *et al.*, 1968), the profile for BsPFK was essentially hyperbolic (Valdez *et al.*, 1989; Byrnes *et al.*, 1994). Table 1 presents the kinetic parameters for the five mutant PFKs as well as the two wild-type enzymes. The k_{cat}, K_m^{ATP}, and $K_m^{Fru\text{-}6P}$ values for the two BsPFK mutants (E161Q and E161A) are similar to the values for wild-type BsPFK. The three EcPFK mutants (Q161E, Q161R, and Q161A) have

Table 1: Steady-State Kinetic Parameters for Wild-Type and Mutant PFKs[a]

enzyme	k_{cat} (s^{-1})	K_m^{ATP} (μM)	$K_m^{Fru\text{-}6P}$ (μM)[b]	$S_{1/2}^{Fru\text{-}6P}$ (mM)[c]	n
WT BsPFK	206 ± 35	70 ± 3	36 ± 2	NA	1.0
Bs E161Q	208 ± 7	112 ± 8	35 ± 3	NA	1.0
Bs E161A	168 ± 12	123 ± 6	41 ± 3	NA	1.0
WT EcPFK	124 ± 2	55 ± 2	NA	0.35 ± 0.01	3.9 ± 0.2
Ec Q161E	126 ± 2	77 ± 4	NA	1.87 ± 0.04	5.6 ± 0.2
Ec Q161R	133 ± 4	47 ± 4	NA	0.91 ± 0.03	5.0 ± 0.4
Ec Q161A	134 ± 2	55 ± 3	NA	0.92 ± 0.02	4.4 ± 0.3

[a] The kinetic parameters were obtained by fitting substrate saturation data to either the Michaelis–Menten equation or the Hill equation. k_{cat}, the catalytic rate constant; K_m^{ATP}, the ATP concentration at half-maximal velocity; $K_m^{Fru\text{-}6P}$ and $S_{1/2}^{Fru\text{-}6P}$, the Fru-6P concentration at half-maximal velocity for Fru-6P saturation data fit to the Michaelis–Menten and Hill equations, respectively; n, the Hill coefficient; NA, not applicable. K_m^{ATP} was obtained in the presence of saturating [Fru-6P], and $K_m^{Fru\text{-}6P}$ was obtained in the presence of saturating [MgATP]. [b] $K_m^{Fru\text{-}6P}$ is appropriate only for BsPFK and its mutants, which follow Michaelis–Menten Fru-6P saturation kinetics. [c] $S_{1/2}^{Fru\text{-}6P}$ is appropriate only for EcPFK and its mutants.

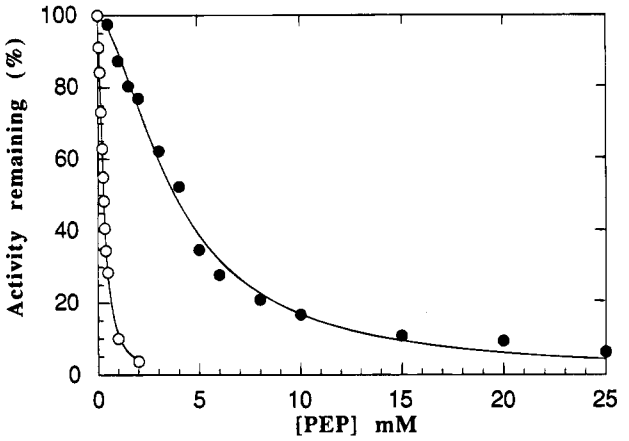

FIGURE 3: PEP Inhibition of *B. stearothermophilus* PFK (○) and *E. coli* PFK (●). The Fru-6P concentration was 0.3 mM for BsPFK and 1.5 mM for EcPFK. The MgATP concentration was 1.0 mM for both.

kinetic parameters similar to those for wild-type EcPFK but show some variation in the sensitivity and cooperativity of their responses to Fru-6P (Table 1). The largest difference in $S_{1/2}^{Fru\text{-}6P}$ value, 6-fold, is seen between the Q161E mutant and wild type. The increase observed in this case could be due to long-range electrostatic repulsion between the carboxylate group of the newly introduced glutamate residue and the phosphate of Fru-6P. The 3-fold increase in $S_{1/2}^{Fru\text{-}6P}$ for the Q161R or Q161A mutants could be caused by minor perturbations of either Fru-6P binding or the kinetic mechanism (Auzat, 1993), or both. These 3–6-fold increases in $S_{1/2}^{Fru\text{-}6P}$ are not large. Thus, alteration of residue 161 does not significantly affect the response of either BsPFK or EcPFK to substrates Fru-6P and MgATP, indicating that this residue is not important for substrate binding and catalysis.

PEP Inhibition. Figure 3 shows that both EcPFK and BsPFK are subject to inhibition by PEP. Their responses to PEP have the same cooperativity (n is 1.6 for BsPFK and 1.7 for EcPFK) but different sensitivities as measured by the half-inhibiting PEP concentration $I_{1/2}^{PEP}$: EcPFK is less sensitive ($I_{1/2}^{PEP}$ is 3.8 mM) in its response to increasing PEP concentration than is BsPFK ($I_{1/2}^{PEP}$ is 0.28 mM). Table 2

Table 2: PEP Inhibition of Wild-Type and Mutant PFKs[a]

enzyme	[Fru-6P] (mM)[b]	$I_{1/2}^{PEP}$ (mM)	n
WT BsPFK	0.3	0.28 ± 0.01	1.6 ± 0.1
Bs E161Q	0.3	3.9 ± 0.1	3.0 ± 0.2
Bs E161A	0.3	0.70 ± 0.01	2.6 ± 0.1
WT EcPFK	1.5	3.8 ± 0.2	1.7 ± 0.2
Ec Q161E	5.0	6.3 ± 0.6	2.4 ± 0.5
Ec Q161R	2.5	9.8 ± 0.2	3.7 ± 0.2
Ec Q161A	5.0	no inhibition	

[a] $I_{1/2}^{PEP}$, the PEP concentration at half-maximal (50%) inhibition; n, the Hill coefficient. The values for $I_{1/2}^{PEP}$ and n were obtained by fitting data from activity versus PEP concentration plots to the Hill equation. [b] The [Fru-6P] used was a saturating amount chosen by inspection of individual activity versus [Fru-6P] plots. The [MgATP] was 1 mM for all assays.

presents the values of $I_{1/2}^{PEP}$ and n for the five mutant and the two wild-type enzymes. Mutation of Glu-161 into Gln or Ala decreases the sensitivity and increases the cooperativity of the response of BsPFK to PEP, suggesting that residue 161 is indeed involved in PEP inhibition of BsPFK. Surprisingly, mutation of Glu to the similarly sized Gln, which removes the negative charge but preserves some hydrogen-bonding ability, is more disruptive for BsPFK than the Glu to Ala mutation that suppresses all hydrogen-bonding ability (Table 2). The reason for the lower PEP sensitivity of the E161Q mutant is not clear but could be due to subtle restructuring of the 6F loop. In EcPFK, mutation of Gln-161 to a charged residue, either Glu or Arg, also decreases the sensitivity and increases the cooperativity of the response to PEP, but not drastically. A positive charge on residue 161 is somewhat more disruptive than a negative charge. However, the Gln to Ala mutation that removes all hydrogen-bonding ability completely abolishes PEP inhibition of EcPFK. Therefore, PEP inhibition of EcPFK requires that residue 161 have some hydrogen-bonding ability. This is not the case for BsPFK, suggesting that residue 161 participates in PEP inhibition of both enzymes but plays a different structural role in each.

GDP Activation of EcPFK and Its Mutants. Figure 4 shows that the activation of wild-type EcPFK by GDP involves an increase in the affinity for Fru-6P, a disappearance of the cooperativity of Fru-6P saturation, and a 10–15% increase in the catalytic rate constant k_{cat} (Blangy *et al.*, 1968; Deville-Bonne *et al.*, 1991). At a fixed Fru-6P concentration equal to $S_{1/2}^{Fru\text{-}6P}$ and a saturating MgATP concentration, the activation of EcPFK by GDP is hyperbolic (inset of Figure 4), and the concentration of GDP needed for half-maximal activation K_{act}^{GDP} can be used to quantitate the sensitivity of GDP activation. All three mutants of EcPFK, Q161E, Q161R, and Q161A, show a hyperbolic Fru-6P saturation in presence of GDP. The slight increase in k_{cat} (10–17%) is similar to that of the wild-type enzyme, and the sensitivities to GDP activation, as measured by K_{act}^{GDP}, are also unmodified (Table 3). Thus, the mutations at position 161 do not alter the mechanism by which GDP activates EcPFK.

Activation by GDP and Saturation by Fru-6P of BsPFK and Its Mutants in the Presence of PEP. Unlike EcPFK, BsPFK is not subject to GDP activation to any significant extent even at nonsaturating Fru-6P concentration. In the presence of PEP, however, GDP will relieve the PEP inhibition of BsPFK and thus appear as an activator. Therefore, GDP activation of BsPFK had to be analyzed

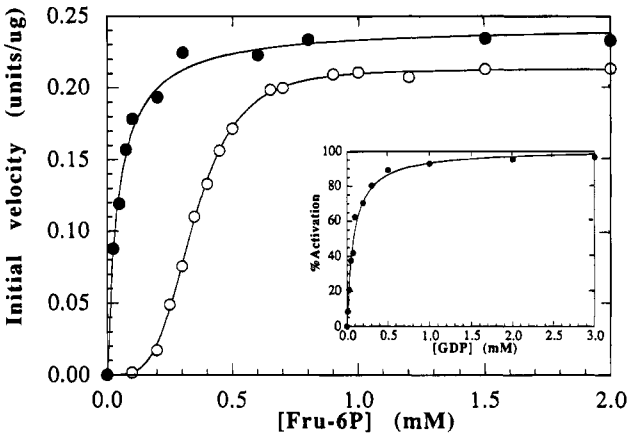

FIGURE 4: GDP Activation of *E. coli* PFK. Fru-6P saturation curves in the absence (○) or presence (●) of 2 mM GDP, showing activation by GDP. The MgATP concentration was 1.0 mM. (Inset) Percent activation versus GDP concentration. The Fru-6P concentration was equal to $S_{1/2}^{Fru\text{-}6P}$ (0.35 mM), and the MgATP concentration was saturating at 1.0 mM.

Table 3: Activation of EcPFK and Its Mutants by GDP[a]

enzyme	k_{cat} (s^{-1})	% activation	K_{act}^{GDP} (μM)
WT EcPFK	141 (124)	14	84 ± 7
Ec Q161E	145 (126)	15	51 ± 12
Ec Q161R	147 (133)	10	84 ± 23
Ec Q161A	157 (134)	17	95 ± 12

[a] k_{cat}, the catalytic rate constant for the enzyme activated by 2 mM GDP (the number in parentheses is the k_{cat} value for the enzyme in the absence of GDP); K_{act}^{GDP}, the concentration of GDP at half-maximal activation. The values for k_{cat} and the % activation were obtained from plots such as the one in Figure 4. K_{act}^{GDP} values were obtained from plots such as the one in Figure 4 (inset). The [MgATP] was 1.0 mM in all assays. In determining K_{act}^{GDP}, the [Fru-6P] was equal to the $S_{1/2}^{Fru\text{-}6P}$ value. See the text for further details.

through its antagonism of PEP inhibition. BsPFK and its mutants were first inhibited by PEP at a concentration equal to the $I_{1/2}^{PEP}$ value for each enzyme (Table 2), and then the ability of GDP to activate the PEP-inhibited enzyme was measured. Figure 5A shows the activation profile obtained for wild-type BsPFK; it is hyperbolic and yields a K_{act}^{GDP} of 122 ± 11 μM. The two mutant enzymes E161Q and E161A also show hyperbolic activation profiles, but their activation constants K_{act}^{GDP} are 4−5-fold lower than that of wild-type BsPFK, revealing an increased sensitivity to GDP activation (Table 4). The mutants E161Q and E161A thus appear to be both less sensitive to PEP inhibition and more sensitive to GDP activation than wild-type BsPFK, which is qualitatively expected from the antagonism between PEP and GDP suggested by both the concerted model and the crystal structure of BsPFK (Schirmer & Evans, 1990).

PEP inhibits BsPFK by causing the Fru-6P saturation to become cooperative and by increasing the half-saturating Fru-6P concentration $S_{1/2}^{Fru\text{-}6P}$ (Valdez *et al.*, 1989). The Fru-6P saturation of wild-type BsPFK in the presence of PEP at a concentration equal to its $I_{1/2}^{PEP}$ gives n = 1.6 and $S_{1/2}^{Fru\text{-}6P}$ = 340 ± 10 μM (Figure 5B), as compared to n = 1 and $K_m^{Fru\text{-}6P}$ = 36 μM in absence of PEP (Table 1). In the presence of a PEP concentrations equal to their $I_{1/2}^{PEP}$ values, the Fru-6P saturations of the mutants E161Q and E161A were also cooperative with values of $S_{1/2}^{Fru\text{-}6P}$ similar to that of wild-type, but with Hill coefficients significantly larger (Table 4). The decrease in the affinity for Fru-6P of the

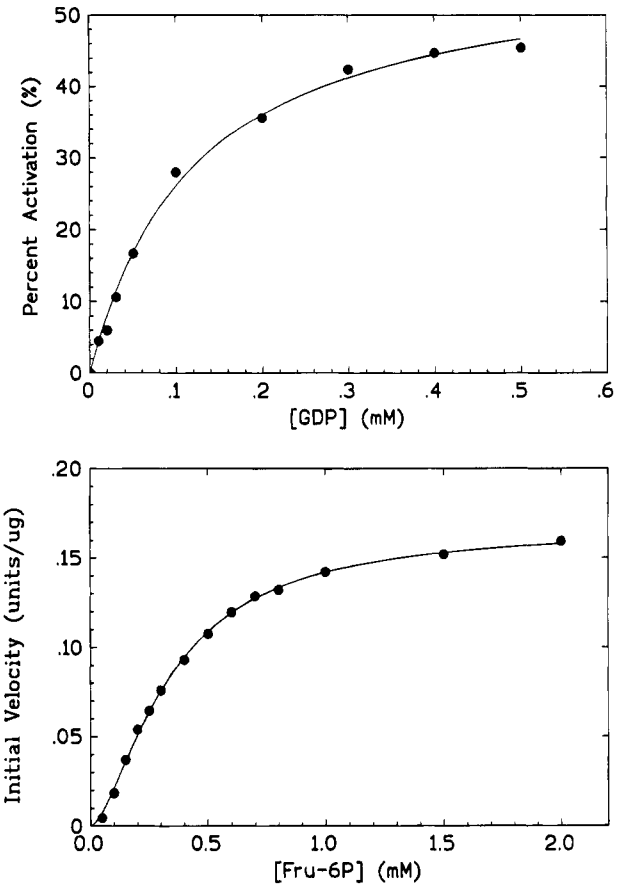

FIGURE 5: Activation of PEP-inhibited *B. stearothermophilus* PFK. The enzyme was inhibited to 50% by PEP at a concentration equal to its $I_{1/2}^{PEP}$ (0.28 mM). (A, top) Percent activation versus GDP concentration, showing the ability of GDP to activate the PEP-inhibited enzyme to 100%. The Fru-6P concentration was 0.3 mM. (B, bottom) Effect of increasing Fru-6P concentration on the activity of the PEP-inhibited enzyme. The MgATP concentration was 1.0 mM in both panels A and B.

Table 4: Activation of BsPFK and Its Mutants by GDP and Fru-6P[a]

enzyme	K_{act}^{GDP} (μM)	$S_{1/2}^{Fru\text{-}6P}$ (mM)	n
WT BsPFK	122 ± 11	0.34 ± 0.01	1.58 ± 0.05
Bs E161Q	23 ± 1	0.28 ± 0.01	3.0 ± 0.1
Bs E161A	29 ± 2	0.28 ± 0.01	2.8 ± 0.2

[a] The kinetic parameters in this table describe the activation of PEP-inhibited BsPFK and its mutants by GDP and Fru-6P. K_{act}^{GDP}, the GDP concentration at half-maximal activation obtained from plots such as the one in Figure 5A; $S_{1/2}^{Fru\text{-}6P}$, the Fru-6P concentration at half-maximal velocity obtained from plots such as the one in Figure 5B; n, the Hill coefficient for the Fru-6P saturation curve. The PEP concentration was equal to its $I_{1/2}^{PEP}$ value. The [MgATP] was 1.0 mM in all assays. In determining K_{act}^{GDP}, the [Fru-6P] was 0.3 mM.

E161Q and E161A mutants caused by PEP is thus the same as for wild-type BsPFK, but their Fru-6P saturations are more cooperative. Remarkably, for each of the three enzymes, i.e., wild-type BsPFK and mutants E161Q and E161A, the cooperativity of the Fru-6P saturation at a fixed PEP concentration equal to $I_{1/2}^{PEP}$ (Table 4) is very close to the cooperativity of its PEP inhibition at a fixed Fru-6P concentration equal to $S_{1/2}^{Fru\text{-}6P}$ (Table 2). This *symmetry* between PEP inhibition and Fru-6P saturation for BsPFK and its mutants strongly suggests that the two processes occur through a common structural pathway, as described in the classical concerted mechanism (Blangy *et al.*, 1968).

DISCUSSION

Using steady-state kinetics, we have studied three mutants of *E. coli* PFK, Q161E, Q161R, and Q161A, and two mutants of *B. stearothermophilus* PFK, E161Q and E161A, in order to better understand the role of residue 161 in the allosteric regulation of the two wild-type enzymes. The kinetic parameters obtained for the mutant enzymes (Table 1) are similar to those for their respective wild-type enzymes. Thus, residue 161 is not directly involved in the binding of substrates or in catalysis. However, our results also show that the hydrogen-bonding ability of residue 161 is critical for PEP inhibition of EcPFK. The mutant Q161A is insensitive to PEP (Table 2). Thus, the stability of the inactive T-state of EcFPK is severely affected by the removal of the hydrogen bonds made by residue 161.

Like Gln-161 of EcPFK, Glu-161 of BsPFK is also involved in the allosteric transition but is not a crucial residue. The BsPFK mutants E161Q and E161A are inhibited by PEP and activated by GDP, albeit somewhat less well than wild type. Both mutants are simultaneously less sensitive to PEP inhibition (Table 2) and more sensitive to GDP activation (Table 4). These opposite effects indicate that inhibition and activation of BsPFK are controlled by the same structural transition between the R- and T-states, as proposed by Schirmer and Evans (1990). The similar cooperativity of both PEP inhibition (Table 2) and Fru-6P saturation of BsPFK and its mutants in the presence of PEP (Table 4) also points to the existence of a unique R-to-T allosteric transition. These results are consistent with the "classical" two-state concerted mechanism of allosteric regulation (Monod et al., 1965).

X-ray crystallography has suggested that Glu-161 contributes significantly to the stability of the inactive T-state of BsPFK by forming a salt bridge with Arg-243 and interacting through a water molecule with Arg-252 of the neighboring subunit (Schirmer & Evans, 1990). The decreased sensitivity of the BsPFK mutants to PEP inhibition and their increased sensitivity to GDP activation indicate that removing either the negative charge of residue 161 or suppressing its hydrogen-bonding ability destabilizes the T-state only moderately. Thus, the equilibrium between the R- and T-states of BsPFK probably involves several other interactions, among which are those between Arg-72 and Glu-241 in the T-state and between Arg-162, Arg-243, and Fru-6P in the R-state [Figure 1 and Schirmer and Evans (1990)].

The overall behavior of EcPFK is much more complex than that of BsPFK in the sense that EcPFK is markedly cooperative in absence of any allosteric effector (Blangy *et al.*, 1968). This "natural" cooperativity of EcPFK is not related to residue 161 being Gln instead of Glu. Indeed, the E161Q mutant of BsPFK still follows an hyperbolic saturation, and the Q161E mutant of EcPFK still follows a sigmoidal saturation [even slightly more sigmoidal than that of wild-type, (Table 1)].

Two of our results in particular show that, in contrast to BsPFK, the behavior of EcPFK cannot be described by the simple concerted model of allosteric regulation with a unique transition between two states R and T (Monod *et al.*, 1965; Blangy *et al.*, 1968). First, it is clear that allosteric inhibition by PEP can be decoupled from Fru-6P cooperativity. The Q161A mutant of EcPFK is insensitive to PEP inhibition (Table 2) but is still as cooperative as the wild-type enzyme, with a Hill number of 4.4 (Table 1). Several modified EcPFK enzymes have been reported to be insensitive to PEP inhibition but nevertheless have sigmoidal Fru-6P saturation kinetics (Le Bras *et al.*, 1982; Serre *et al.*, 1990; Byrnes *et al.*, 1995). Thus, Fru-6P cooperativity and allosteric PEP inhibition of EcPFK are not related to the same structural transition and likely occur via different pathways altogether. This is not to say that there is no overlap between the two processes since the active sites are involved in both, and mutations of some residues at the active site can affect both PEP inhibition and Fru-6P cooperativity, as reported for Arg-72 or Thr-125 (Auzat *et al.*, 1994a).

The second result showing that the properties of EcPFK are incompatible with a unique two-state allosteric transition is the decoupling between PEP inhibition and GDP activation. Indeed, although the Q161A mutant is insensitive to PEP (Table 2), it is still activated by GDP (Table 3). Certain mutations in the effector site of EcPFK also lead to a partial or total decoupling between PEP inhibition and GDP activation (Lau *et al.*, 1987; Auzat *et al.*, 1994b). PEP probably inhibits EcPFK by inducing a transition from the active R-state into the inactive T-state (Schirmer & Evans, 1990). Therefore, mutants that are insensitive to PEP are unable to isomerize into the T-state: they remain in the R-state and should be insensitive to GDP activation. However, the EcPFK mutant Q161A is activated by GDP (Table 3) despite its insensitivity to PEP inhibition (Table 2). This indicates that GDP activation in the absence of PEP does not involve residue 161 and proceeds through a structural pathway different from that of PEP inhibition. The activation of EcPFK by GDP would thus depend not only on the R-to-T transition, but (at least in part) also on a change within the R-state. There would be two mechanisms for GDP activation of EcPFK: one, obeyed in absence of PEP, that involves only the R-state (and not Gln161) and another, obeyed in presence of PEP, that involves a GDP-induced shift towards the R-state. The latter mechanism would be similar to the one operating in BsPFK (Schirmer & Evans, 1990).

Finally, a comparison between rabbit muscle PFK and the bacterial enzymes illustrates how the role of a given residue can change with evolution. Rabbit muscle PFK is about twice the size of bacterial PFK and has clear N- and C-half internal homology, strongly suggesting that its gene arose from a procaryotic progenitor by duplication and divergence (Poorman *et al.*, 1984). Gln-200 in rabbit muscle PFK corresponds to residue 161 of bacterial PFK. Mutations of Gln-200 strongly alter the Fru-6P cooperativity and homotropic regulation of the enzyme by ATP but only moderately affect its heterotropic regulation by effectors fructose 2,6-bisphosphate and citrate (Li *et al.*, 1993). In the current study, we show that residue 161 is important for heterotropic regulation of the bacterial PFKs by PEP but is not important for Fru-6P cooperativity *per se*. Comparing the role of Gln-200 in the regulation of RMPFK to the role of residue 161 in the regulation of bacterial PFKs, one can see that, in the course of evolution from bacteria to mammals, the residue has lost its importance in regulation by allosteric effectors and has become critical for allosteric inhibition by ATP and the control of Fru-6P cooperativity. Thus, after gene duplication and divergence, the residue has acquired a new regulatory role (Poorman *et al.*, 1984).

ACKNOWLEDGMENT

We are grateful to Dr. Philip R. Evans for giving us the recombinant *ecpfk*/pUC18 plasmid and for helpful discussions at the inception of this work.

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BI9501489