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Role of Glycine 212 in the Allosteric Behavior of Phosphofructokinase from *Bacillus stearothermophilus*†

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ABSTRACT: Crystallographic studies indicate that the loop between α-helix 8 and β-strand H (the 8H loop) which borders the effector site of *Bacillus stearothermophilus* phosphofructokinase (BsPFK) is involved in the allosteric mechanism of the enzyme [Schirmer, T., and Evans, P. R. (1990) *Nature 343*, 140−145]. The residue at one end of this loop, glycine 212, has been proposed to be a pivot about which the loop hinges. Using site-directed mutagenesis, glycine 212 was replaced with valine (G212V). Steady-state kinetic analysis and ligand binding studies on the altered and native PFKs showed that the G212V substitution resulted in discernible changes at the effector site. The mutated PFK required a 3-fold higher concentration of the allosteric inhibitor phosphoenolpyruvate than did the native enzyme to cause the same level of inhibition. The altered PFK had a 2-fold higher dissociation constant for the allosteric activator GDP than the wild-type enzyme. More importantly, whereas the native PFK was fully activated by 1 mM GDP from its PEP-inhibited T-state, the altered enzyme was only marginally activated. On the other hand, the G212V mutation resulted in no changes at the catalytic site of BsPFK. The catalytic rate constant k_{cat} remained unchanged. The altered PFK had the same K_m values for ATP and fructose-6-phosphate (Fru-6-P) as did the wild-type enzyme. Furthermore, starting from the same PEP-inhibited T-state, both enzymes gave identical sigmoidal responses to increasing Fru-6-P concentration, indicating that Fru-6-P can activate both to the R-state.

The activity of an allosteric enzyme can be regulated homotropically through the binding of a cooperative substrate in the active site and heterotropically by the binding of allosteric effectors in the effector site [reviewed by Perutz (1990)]. An example of such regulation is seen in the glycolytic enzyme phosphofructokinase (PFK,[1] EC 2.7.1.11), which catalyzes the ATP-dependent phosphorylation of fructose-6-phosphate in the first committed step of glycolysis [reviewed by Evans (1992)]. PFK from *Bacillus stearothermophilus* (BsPFK) is a tetramer of identical subunits having 319 residues each. X-ray diffraction analysis reveals that the BsPFK tetramer can be viewed as a dimer of rigid dimers A:B and C:D (Schirmer & Evans, 1990; Evans, 1992). Each subunit is composed of a large and a small domain, with the active site located in a cleft between the two domains. The ATP binding region of the active site is comprised of residues from the large domain only, whereas the fructose-6-phosphate (Fru-6-P) binding region is comprised of residues from both the small and large domains as well as residues across the dimer−dimer interface. There are four active and four effector sites in the tetramer. Each effector site lies in a deep cleft between the two subunits of the rigid A:B or C:D dimer, and both the allosteric inhibitor, phosphoenolpyruvate (PEP), and the allosteric activator, ADP (or GDP), bind to the same site.

In the presence of PEP, BsPFK is in a low-activity T-state and can be converted to a high-activity R-state upon addition of GDP. Schirmer and Evans (1990) have elucidated the structural basis for the allosteric transition of BsPFK from a comparison of the crystal structures of the enzyme obtained under two different conditions: (1) in the presence of inhibitor 2-phosphoglycolate, an analog of PEP (Schimer & Evans, 1990), and (2) in the presence of ADP (Evans & Hudson, 1979; Evans et al., 1981). During the transition from the T-state to the R-state, the A:B and C:D dimers rotate 7° relative to each other. This rotation is coupled with a coordinated movement of two loops, the 8H and 6F loops, located between the effector and active sites (Figure 1). At the effector site, the major movement involves a change in the position of the 8H loop, which lines one edge of the effector cleft. In the absence of effector, the cleft is open. Binding of the smaller molecule PEP closes the cleft, shifting the enzyme to its T-state. When the larger ADP (or GDP) is bound in the cleft, the 8H loop is pushed away from the

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[1] Abbreviations: PFK, phosphofructokinase; BsPFK and EcPFK, PFKs of *Bacillus stearothermophilus* and *Escherichia coli*, respectively; *pfk*, gene encoding PFK; G212V, BsPFK mutated at glycine 212 to valine; kb, kilobase pair; RF, replicative form of M13 DNA; X-gal, 5-bromo-4-chloro-3-indoyl-β-D-galactoside; IPTG, isopropyl-β-D-thiogalactoside; YT, medium containing yeast extract and tryptone; Fru-6-P, fructose-6-phosphate; Fru-1,6-P_2, fructose-1,6-bisphosphate; PEP, phosphoenolpyruvate; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; R and T, high-activity and low-activity states of allosteric enzymes, respectively; MWC, Monod−Wyman−Changeux model for allosteric proteins; 6F (or 8H) loop, loop region between α-helix 6 and β-strand F (or α-helix 8 and β-strand H) of BsPFK; $S_{0.5}$, substrate concentration at half-saturation obtained by fitting the saturation data to the Hill equation; $K_{T(PEP)}$, dissociation constant of PEP from the T-state of BsPFK; $K_{i(GDP)}$, dissociation constant (or competitive inhibitor constant) of GDP from the catalytic site; $K_{d(GDP)}$, dissociation constant of GDP from the effector site.

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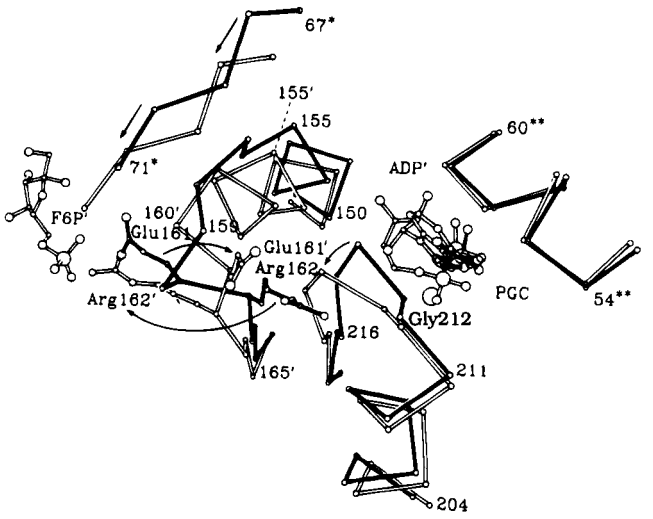

FIGURE 1: 8H loop movement of BsPFK during the allosteric transition between its T-state (solid lines) and R-state (open lines). Courtesy of Dr. P. R. Evans and permission from *Nature* [Shirmer, T., & Evans, P. R. (1990) *Nature 343*, 140−145]. Copyright (1990) Macmillan Magazines Limited.

effector site, where it contacts the 6F loop and transmits a structural change across the dimer−dimer interface to the active site. The 6F loop, which is comprised of residues threonine 156 to arginine 162, becomes reorganized during this process. In response to GDP binding, arginine 162, which points away from the substrate binding site in the T-structure, swings back into the active site where it binds the phosphate group of Fru-6-P. These conformational changes describing the allosteric transition are consistent with the concerted two-state allosteric model of Monod, Wyman, and Changeux (MWC model; Monod et al., 1965).

Glycine 212 acts as one of the pivots on which the 8H loop hinges in response to the binding of PEP and ADP (or GDP). It is conserved between BsPFK and the PFK from *Escherichia coli* (EcPFK). In this study, glycine 212 has been mutated to valine to give residue 212 a bulkier side chain. Using steady-state kinetics and equilibrium dialysis, we have studied how this substitution affects the response of the enzyme to effectors.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. A 2.5 kilobase *Eco*RI/*Cla*I fragment containing the *bspfk* gene that had been cloned into pBR322 (French et al., 1987) was subcloned into the *Eco*RI/*Acc*I sites of M13mp18 for mutagenesis. Competent DH5αF′ *E. coli* cells (Table 1) were transformed with the recombinant RF DNA and then plated on a YT agar plate containing 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) and isopropyl-β-D-galactoside (IPTG). The supernatant from a clear plaque harboring the *bspfk* insert was used to infect BW313 strain *E. coli* cells (Table 1). Uracil-containing single-stranded DNA was prepared from the infected BW313 cells and used as template for the *in vitro* mutagenesis as described by Kunkel (1985). The mutagenic oligonucleotide was designed to change the codon at amino acid position 212 from that for glycine (GCC) to that for valine (GUU). The M13-infected DH5αF′ transformants were screened for the mutation by (1) plaque hybridization using the mutagenic oligonucleotide labeled with ^{32}P as probe (Seong & Raj-Bhandary, 1987) and (2) partial sequencing of the single-stranded DNA from single positives identified in the plaque hybridization. Single-stranded DNA was prepared from positive transformant, and the entire coding region of the mutated gene was sequenced by the dideoxy method (Sanger, 1977) using a Sequenase kit (United States Biochemical) to verify that no unintentional mutation had been introduced.

Expression and Purification of PFK. The mutated gene was excised from M13mp18 and inserted into the *Eco*RI/*Hind*III sites of the plasmid pUC18. The recombinant plasmid was then transformed into competent cells of a PFK-deficient *E. coli* strain (DF1020, Table 1) for expression. The transformed DF1020 cells were grown to stationary phase in liquid culture, and the cells were pelleted, resuspended in buffer A (50 mM Tris-HCl, pH 7.4, 1 mM DTT, 1 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride), and sonicated. PFK was purified from the resulting crude extract by a procedure that involved heat treatment at 70 °C followed by affinity chromatography on a Cibacron blue 3GA column (Valdez et al., 1989). After purification, the enzyme solution was concentrated by dialysis against 50% glycerol in buffer A. Preparations of the native and G212V BsPFKs were each shown to be pure by electrophoresis in a 12% SDS-polyacrylamide gel stained with Coomassie brilliant blue. Protein concentrations were determined by the Bradford method using the Bio-Rad (Richmond, CA) protein assay reagent. PFK activity was expressed in units of μmol of Fru-1,6-P_2 formed/min. The specific activities of the purified native BsPFK and the altered enzyme were 286 and 249 units/mg, respectively.

PFK Activity Assay. BsPFK activity was determined by coupling the production of Fru-1,6-P_2 to the oxidation of NADH at 30 °C. The assay mixture 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 containing 1 mM creatine phosphate and 10 μg/mL creatine phosphokinase was used to prevent ADP-mediated effects In studying the effect of GDP, this regenerating system was not used. The oxidation of NADH was monitored at 340 nm using a Hitachi UV-2000 spectrophotometer. Relative activity is the ratio of measured activity to the maximal activity of each enzyme in the absence of any effector.

Analysis of Kinetic Data. Initial velocity data for both the native BsPFK and the altered enzyme in the absence of effector were fit to the Michaelis−Menten equation to obtain K_m and k_{cat} values for each enzyme. In the presence of PEP or both PEP and GDP, the initial velocity data were fit to the Hill equation to calculate the Hill number n_H and the half-saturation value $S_{1/2}$. The ATP saturation data from GDP inhibition studies were plotted graphically by Lineweaver−Burk plots. The slope of each line was then determined and plotted against GDP concentration to obtain $K_{i(GDP)}$, the dissociation constant of GDP at the active site.

For determining the dissociation constant of PEP from the T-state of BsPFK, $K_{T(PEP)}$, the initial velocity data were fit to Equation 1, which is derived from MWC theory (Monod et al., 1965)

$$[(V'_{max} - v)/v]^{1/n} = (L_\alpha^{1/n}/K_T)[PEP] + L_\alpha^{1/n} \quad (1)$$

where n is the number of PEP binding sites (four for BsPFK),

Table 1: Genotypes of *E. coli* Strains Used in This Study

E. coli strain	genotype	reference
DH5αF′	F′, *end*A1, *hsd*R17, (r_K^-, m_K^+), *sup*E44, *thi*-1, *rec*A1, *gry*A96, *rel*A1, λ^-, ϕ80d*lac*Z$^-$, M15(*lac*ZYA*arg*F)U169	Hanahan (1983)
BW313	*Hfr*, *lys*A$^-$, *dut*, *ung*, *thi*-1, *rec*A, *spo*T1	Kunkel (1987)
DF1020	*pro*-82, *pfk*B201, *rec*A56, (*rha-pfk*A)200, *end*A1, *hsd*R17, *sup*E44, *thi*-1	Daldal (1983)

v is the initial velocity, V'_{max} is the maximum velocity that can be reached at the fixed concentration of Fru-6-P, and L_α is an apparent allosteric constant. Using eq 1, $K_{T(PEP)}$ can be obtained from the negative reciprocal of the intercept on the abscissa in a plot of $[(V'_{max} - v)/v]^{1/4}$ vs [PEP].

Equilibrium Dialysis. Direct binding of Mg^{2+}•GDP to the native and altered enzymes was studied using the equilibrium dialysis method described by Blangy (1971). A five-cell rotating equilibrium dialyzer (Spectrum, Houston, TX) was used to perform the experiment. The volume of each cell was 1.0 mL. [^{3}H]GDP at 38.6 Ci/mmol was obtained from DuPont-NEN and diluted with unlabeled GDP before use. The total GDP concentration varied from 0.4 to 50 μM for the native BsPFK and 1 to 100 μM for the G212V enzyme. The concentration of protein used in the experiment was 0.15 mg/mL for the native BsPFK and 0.14 mg/mL for the G212V enzyme. Dialysis was carried out in 50 mM Tris•HCl, pH 8.2, 1 mM $MgCl_2$, and 2 mM DTT for 14−16 h at 4 °C. After dialysis, an aliquot of 0.1 mL was removed from the sample compartment and mixed with 5 mL of scintillation solution (Ecolite, ICN). The radioactivity of the sample was counted in a Beckman liquid scintillation system (Beckman Instruments, Inc., Fullerton, CA). A control without protein was run to verify that the nucleotide added in one compartment equilibrated completely with the other through the dialysis membrane. Equilibrium binding data were analyzed using the Scatchard equation (Scatchard, 1949) by plotting $r/[GDP]_{free}$ vs r, where r is the ratio of the concentration of bound ligand to the enzyme concentration and $[GDP]_{free}$ is the free GDP concentration. The slope of the curve is equal to $-1/K_{d[GDP]}$, and the intercept on the abscissa gives the number of GDP binding sites per enzyme molecule. A straight line should be obtained if all the binding sites have the same intrinsic dissociation constant and the binding is independent from binding to other sites (Scatchard, 1949). All curve fitting was performed by linear or nonlinear regression using the program INPLOT 4.0 (GraphPad, Inc., San Diego, CA).

RESULTS

Steady-State Kinetics of G212V PFK in the Absence of Effector. It has been shown that BsPFK follows hyperbolic kinetics with respect to its two substrates Fru-6-P and ATP (Valdez et al., 1989). In the absence of effectors, G212V BsPFK exhibited a nearly identical Fru-6-P saturation profile (Figure 2A), and essentially the same $K_{m(ATP)}$ and $K_{m(Fru\text{-}6\text{-}P)}$ values as those of the native enzyme (Table 2). Thus, the G212V substitution did not affect the affinity of the enzyme for either substrate. Both the native and altered BsPFKs had similar values of k_{cat}, indicating that the mutation did not change the catalytic activity of the enzyme.

Sensitivity of G212V PFK to Inhibition by PEP. Phosphoenolpyruvate is an allosteric inhibitor of BsPFK. In the presence of PEP, the enzyme favors the T-state and its affinity for Fru-6-P is reduced. This causes the Fru-6-P saturation profile to be sigmoidal. To investigate the effects of the G212V substitution on PEP inhibition, we analyzed Fru-6-P saturation kinetics for the altered PFK in the absence

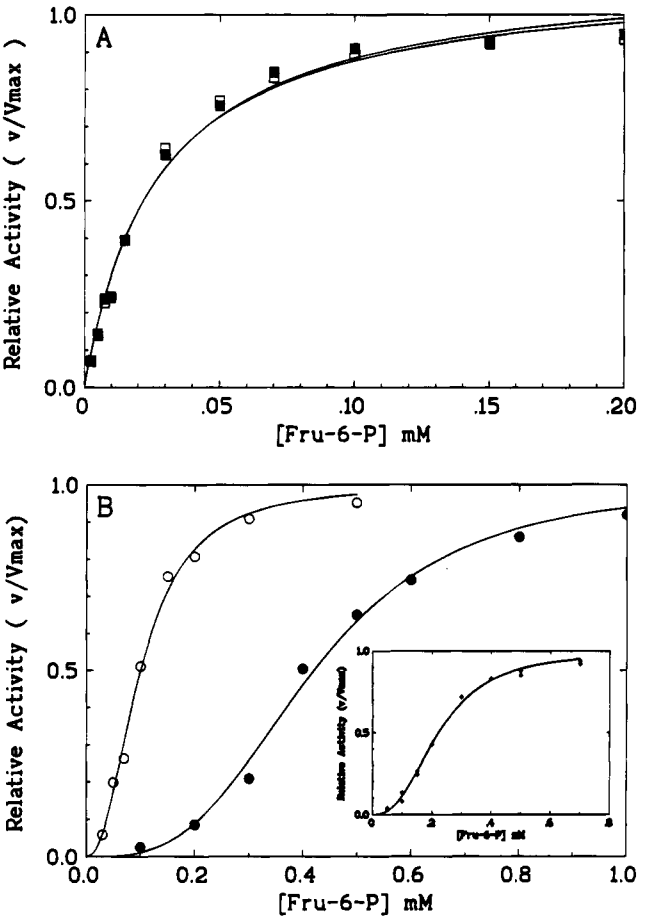

FIGURE 2: (A) Fru-6-P saturation profiles in the absence of PEP. (■) Native BsPFK and (□) G212V BsPFK. (B) Dependence of PFK activity on Fru-6-P concentration in the presence of 0.5 mM PEP. (●) Native BsPFK and (○) G212V BsPFK. The inset shows the Fru-6-P saturation profiles of G212V BsPFK at 1.0 mM PEP (◇) and the native enzyme at 0.3 mM PEP (◆). The activity assay is described under Experimental Procedures. Each assay contained 1.0 mM ATP.

Table 2: Steady-State Kinetic Parameters[a] of the Native and G212V BsPFKs

enzyme	$K_{m(ATP)}$ (μM)	$K_{m(Fru\text{-}6\text{-}P)}$ (μM)	k_{cat} s−1	$K_{i(GDP)}$ (μM)
wild-type	118 + 4	23 + 2	149	1.5
G212V	128 + 7	26 + 2	126	2.2

[a] K_m values with respect to ATP or Fru-6-P were obtained from ATP saturation profiles in the presence of 1 mM Fru-6-P or from Fru-6-P saturation profiles in the presence of 1 mM ATP, respectively. k_{cat} values were measured at saturating substrate concentration for each enzyme. $K_{i(GDP)}$ values were obtained from ATP saturation profiles in the presence of different GDP concentrations. Experiments were performed three to five times. Results are within the error range; ± values are errors estimated by the INPLOT program.

Table 3: Comparison between $K_{T(PEP)}$ Values[a] for the Native BsPFK and the G212V Enzyme at Different Concentrations of Fru-6-P

Fru-6-P (mM)	$K_{T(PEP)}$ (mM) native	$K_{T(PEP)}$ (mM) G212V
0.15	0.16	0.38
0.24	0.33	0.71
0.45	0.42	1.36

[a] $K_{T(PEP)}$ values were obtained from $[(Vmax' - v)/v]^{1/4}$ vs [PEP] plots at different Fru-6-P concentrations.

and presence of PEP and compared the results to those obtained for the native enzyme. Figure 2A shows that in the absence of PEP both the native BsPFK and the altered enzyme gave a hyperbolic response to increasing concentration of Fru-6-P. However, in the presence of 0.5 mM PEP, both enzymes gave sigmoidal responses (Figure 2B). The Hill numbers derived from these curves were 3.14 for the native BsPFK and 2.26 for the G212V enzyme, and their $S_{1/2(Fru-6-P)}$ values were 420 and 100 μM, respectively. Thus, both enzymes showed cooperative behavior in the presence of PEP, but the altered enzyme had a 4 times lower $S_{1/2(Fru-6-P)}$ value than the native enzyme. As shown in the inset of Figure 2B, the sigmoidal responses of these two PFKs were superimposed when the concentration of PEP for the native enzyme was adjusted to 0.3 mM and that for the altered enzyme was 1.0 mM. This observation indicates that the G212V PFK can reach the same level of allosteric inhibition as that of the native enzyme but requires a higher concentration of PEP. Consistent with this observation, values for the dissociation constant of PEP, $K_{T(PEP)}$, from the T-state BsPFK calculated at three different Fru-6-P concentrations were higher for the altered enzyme than the corresponding values for native BsPFK (Table 3).

Sensitivity of T-State G212V PFK to Activation by GDP. GDP activation of BsPFK can only be observed in the presence of inhibitor PEP. As shown in Figure 2B, the native and altered BsPFK species responded to PEP inhibition differently. Comparing GDP activation of the two enzymes in the presence of the same concentration of PEP would be misleading since they are not inhibited to the same extent by PEP (Figure 2B). Therefore, we first brought the enzymes to the same PEP-inhibited state with appropriate PEP concentrations, i.e., 0.3 mM PEP for the native BsPFK and 1 mM for the altered enzyme (inset, Figure 2B), before subjecting them to GDP activation. The results of the GDP activation studies are shown in Figures 3. Fru-6-P saturation profiles for the native BsPFK in the absence and presence of 1 mM GDP showed significant activation as illustrated by the reduction in the Hill number from 2.9 to 1.0 and the decrease in the $S_{1/2\ (Fru-6-P)}$ value from 200 to 40 μM (Figure 3A). However, GDP at a concentration of 1 mM or higher provided little activation of the altered PFK; neither the Hill number nor the $S_{1/2(Fru-6-P)}$ were significantly affected (Figure 3B and Table 4). Therefore, unlike the native BsPFK, the altered enzyme was barely activated by GDP.

The activities measured for both the native and altered BsPFKs in the presence of increasing concentrations of GDP also support the conclusion that GDP cannot activate G212V PFK. Since Fru-6-P is a cooperative substrate for BsPFK in the presence of PEP, comparison between the extents of GDP activation of these two forms of BsPFK would be

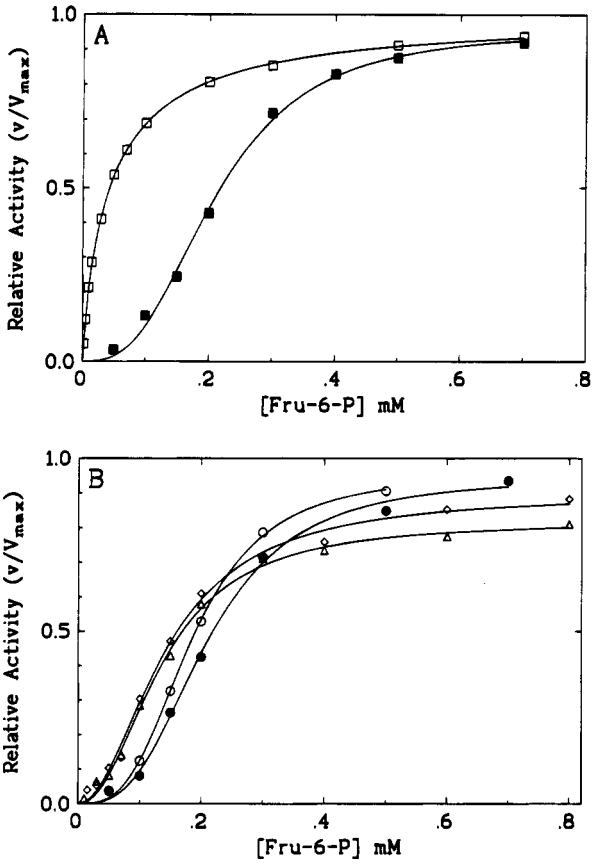

FIGURE 3: Effect of GDP on Fru-6-P saturation in the presence of PEP. Fru-6-P saturation curves for (A) native BsPFK in the presence of (■) 0.3 mM PEP only or (□) 0.3 mM PEP and 1.0 mM GDP and for (B) G212V BsPFK in the presence of (●) 1.0 mM PEP only, (○) 1.0 mM PEP and 1.0 mM GDP, (◇) 1.0 mM PEP and 2.0 mM GDP, or (△) 1.0 mM PEP and 3.0 mM GDP.

Table 4: Effect of GDP on the Activity of Native BsPFK and G212V Enzyme in the Presence of Different Concentrations of PEP

PEP (mM)	GDP (mM)	native n_H[a]	native $S_{1/2}$[a] (mM)	G212V n_H	G212V $S_{1/2}$ (mM)
0.2	0	2.3 + 0.2	0.18	2.7 + 0.1	0.06
0.2	1.0	1.1 + 0.1	0.04	2.5 + 0.1	0.06
0.5	0	3.1 + 0.2	0.42	2.3 + 0.1	0.10
0.5	1.0	1.4 + 0.2	0.14	2.1 + 0.2	0.11
1.0	0	2.8 + 0.1	0.53	2.6 + 0.2	0.22
1.0	1.0	1.2 + 0.1	0.20	3.0 + 0.1	0.19

[a] n_H is the Hill number for Fru-6-P. $S_{1/2}$ is the Fru-6-P concentration at half-maximal velocity in the presence of 1 mM ATP. Analyses were performed three to five times. Error range is described in the footnote of Table 2.

meaningful only at low Fru-6-P concentration. Starting from the same level of PEP inhibition in the presence of an unsaturating concentration of Fru-6-P (0.03 mM), the native enzyme was activated by 2 mM GDP to about 85% of its activity in the absence of PEP. However, the same or higher concentrations of GDP could activate G212V PFK by only 10% (Figure 4). Thus, GDP had little activating effect on the altered enzyme. On the other hand, concentrations of GDP higher than 1 mM (and a saturating concentration of Fru-6-P) inhibited PFK activity for both the native (Valdez et al., 1989; Byrnes et al., 1994) and altered enzymes. Competitive inhibition patterns were observed when ATP saturation profiles were analyzed at 1, 2, and 3 mM GDP and a saturating concentration of Fru-6-P (data not shown).

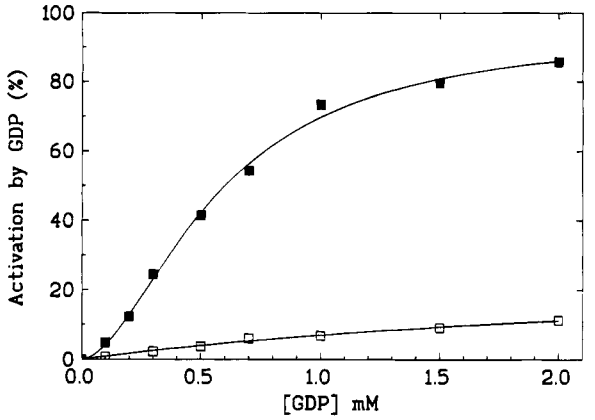

FIGURE 4: Activation by GDP of the native and G212V BsPFKs at 0.03 mM Fru-6-P. (■) Native BsPFK and (□) G212V BsPFK. The percent activation is the activity compared to the activity in the absence of effector at 0.03 mM Fru-6-P. The ATP concentration was 1.0 mM. PEP concentrations were as described in Figure 3.

Values of $K_{i(GDP)}$ were essentially the same for both forms of BsPFK (Table 2). The competitive inhibition patterns indicate that GDP could also bind in the substrate ATP site and that this binding is not affected by the G212V mutation.

Equilibrium Dialysis. To further understand the lack of GDP activation of G212V-mutated PFK, the binding of GDP to the native BsPFK and the altered enzyme was examined by equilibrium dialysis. The results of this analysis reveal several important insights into the way GDP binds to BsPFK. First, the dissociation constants of GDP, $K_{d(GDP)}$, from the native and altered enzymes were 27 and 56 μM, respectively. Since the equilibrium constants for dissociation of GDP from the catalytic site, $K_{i\ (GDP)}$, are much higher (1.5 mM for the native enzyme and 2.2 mM for the G212V enzyme, Table 2), the $K_{d(GDP)}$ values obtained here are due to dissociation from the effector site. The 2-fold higher value of $K_{d(GDP)}$ for the altered BsPFK versus the native enzyme indicates that mutation of glycine to valine at position 212 does not drastically change the affinity of the enzyme for GDP. However, the kinetic studies showed that the altered enzyme was not allosterically activated by GDP. Together, these results suggest that although the G212V PFK can still bind GDP in the effector site, the interactions responsible for converting the enzyme to the active structure are no longer effective. Thus, the G212V mutation blocks the transmission of GDP-mediated conformational signal from the effector site to the catalytic site. Second, unlike EcPFK for which binding of GDP at the effector site is cooperative (Blangy, 1971), we observed in the current work that both native BsPFK and the G212V enzyme showed noncooperative GDP binding profiles (Figure 5A). This suggests that the binding of GDP at one effector site is independent of its binding at another. Finally, combined with the latter results, the value of 4 obtained for the number of GDP binding sites (Figure 5B) indicates that both the native and G212V PFKs have four noninteracting binding sites for GDP. The same number of GDP (or ADP) binding sites per molecule (i.e., four) is observed from the X-ray crystal structure of native BsPFK (Schirmer & Evans, 1990).

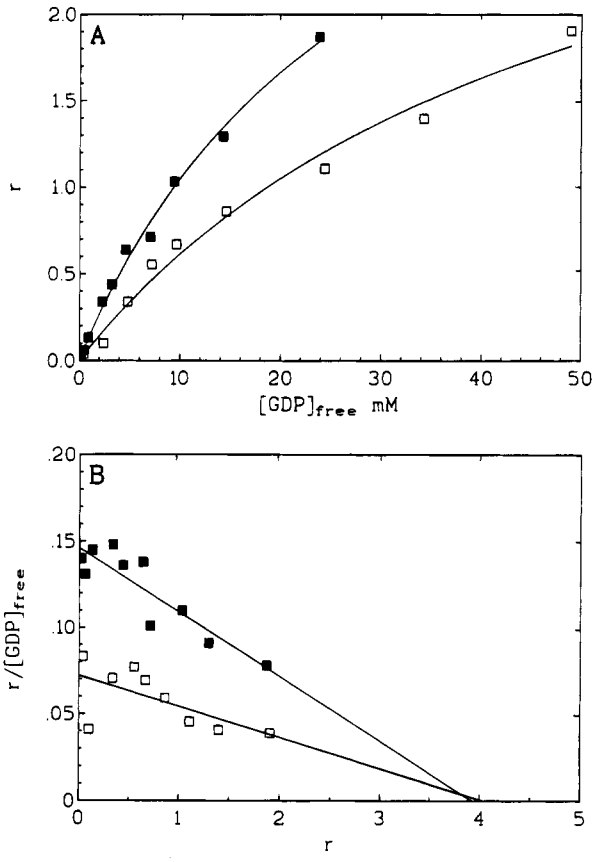

FIGURE 5: Results of equilibrium dialysis of native BsPFK (■) and G212V BsPFK (□) against [^{3}H]GDP. Experimental procedures are described in the text. (A) The ratio (r) of bound GDP to the enzyme concentration, both in micromolar, was plotted against free [GDP]. (B) Scatchard plots of the same data. The dissociation constant K_d of GDP is -1/slope, and the number of GDP binding sites per enzyme molecule is the intercept on the abscissa (the r coordinate).

DISCUSSION

Glycine residues are often located at the flexible turns of protein molecules (Creighton, 1993; Bossemeyer, 1994) where they play an important role in allowing the folding of polypeptide chains into structural motifs that can form binding domains for substrates, cofactors, or regulatory effectors. The major aim of this work has been to examine, using site-directed mutagenesis, the role of the glycine 212 in the allosteric properties of BsPFK. Glycine 212 was mutated to valine, a residue with a significantly bulkier side chain. The mutated BsPFK was compared to the native enzyme in its steady-state kinetics and its ability to bind GDP. The results showed that the mutation of glycine 212 to valine rendered discernible changes in the heterotropic regulatory properties of BsPFK: the altered enzyme required 3-fold higher concentration of PEP than that needed by the native enzyme to reach the same level of inhibition, and it displayed a 2-fold higher $K_{d(GDP)}$ value than did the native enzyme. More significantly, whereas the native BsPFK was fully activated from its PEP-inhibited T-state by GDP, the altered enzyme could barely be activated by GDP at the same or higher concentration (Figures 3 and 4). On the other hand, the G212V mutation did not directly affect the catalytic site: the catalytic constant k_{cat} was unaltered, and the mutated enzyme gave the same K_m values for Fru-6-P and ATP. Furthermore, starting from the same PEP-inhibited state, the native and mutated enzymes were activated to the same extent by Fru-6-P with essentially identical sigmoidal response to the concentration of this substrate (Figure 2B, inset). This suggests that the ability of Fru-6-P to reactivate

the PEP-inhibited enzyme was not altered by G212V mutation.

A comparison between the R- and T-structures of BsPFK shows that the 8H loop moves toward or away from the effector site in response to the binding of PEP or GDP, respectively, at the effector site (Figure 1). This movement transmits the conformational signal of inhibition or activation from the effector site to the catalytic site (Schirmer & Evans, 1990). The 8H loop is composed of residues Gly212, Lys213, Lys214, and His215. The lack of a chiral side chain makes Gly212 the most flexible residue in this loop. A mutation of this residue to valine would be expected to affect the local conformation of the 8H loop. This is suggested by the ϕ and ψ angles of Gly212 which are 88° and 19°, respectively, in the R-state and 101° and 17° in the T-state (Evans, P. Personal communication). These values are in the partially allowed region of the Ramachandran plot (Morris, et al., 1992; Creighton, 1993). The structure of Val212 in the mutated PFK would likely rearrange to be within the partially allowed region. This would in turn cause other minor local rearrangements within the 8H loop. We note that while such rearrangements of local conformation can certainly explain the altered allosteric function of the 8-H loop, the actual structural basis for the concurrent loss of GDP activation and retention of PEP inhibition in G212V PFK is not known.

The roles of the different effector binding residues in the allosteric properties of EcPFK have been throughly dissected using site-directed mutagenesis (Lau et al., 1987; Lau & Fersht, 1989). One of these residues is lysine 213 which, like glycine 212, is located within the 8H loop. An altered EcPFK produced by changing lysine 213 to alanine (K213A) displays properties similar to those of G212V BsPFK: it decreases the affinity for PEP and abolishes GDP-mediated activation. Although the two altered enzymes have similar responses to effectors, the structural bases of their responses are somewhat different. G212V has an enlarged side chain which attenuates the allosteric function of the 8H loop, while K213A has lost the positive charge critical for interaction with the carboxyl group of PEP or the β-phosphate of GDP (Shirakihara & Evans, 1988; Schirmer & Evans, 1990).

It has long been thought that the kinetic properties of BsPFK would be similar to those of EcPFK, which have been extensively studied. However, our studies with respect to the allosteric response of BsPFK to PEP inhibition were difficult to reconcile with Monod's concerted model. Unlike EcPFK which obeys the MWC model in terms of its response to PEP inhibition and GDP activation, i.e., its $K_{T(PEP)}$ and $K_{R(GDP)}$ values are independent of Fru-6-P concentration (Blangy et al., 1968; Lau et al., 1987), BsPFK displayed $K_{T(PEP)}$ values that depended on the Fru-6-P concentration under similar assay conditions [Valdez et al. (1989) and current work]. The G212V enzyme showed a 3−4-fold increase in $K_{T(PEP)}$ as the Fru-6-P concentration increased 3-fold (Table 3).

The crystallographic structure of BsPFK at high resolution offers an ideal guide for the design of site-directed mutagenesis studies on the allosteric control of this enzyme. Furthermore, because of the evolutionary conservation between bacterial and eukaryotic PFKs (Poorman et al., 1984; Hellinga & Evans, 1985), the former can serve as a prototype for studies on the allosteric regulation of the latter (Li et al., 1993).

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