Vol. 156, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1988 Pages 537-542

SITE-DIRECTED MUTAGENESIS AT THE REGULATORY SITE OF FRUCTOSE 6-PHOSPHATE-1-KINASE FROM Bacillus stearothermophilus

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Received August 29, 1988

We have mutated Arg-25, Asp-59 and Arg-211 to alanine; and Asp-59 also to methionine, in fructose 6-phosphate-1-kinase from B. stearothermophilus (designated as RA25, DA59, RA211 and DM59 respectively). All four mutants did not change the affinity of the enzyme for ATP. RA25 has half the affinity for fructose 6-phosphate and exhibits sigmoidicity with respect to this substrate (Hill # = 2.0). DA59 has the same affinity for phosphoenolpyruvate (PEP) as the wild type whereas DM59 has 3-fold the affinity for this modulator and the inhibition is reversed by GDP. RA25 and RA211 are 100-fold less sensitive to PEP inhibition which is not relieved by GDP. It is concluded that Arg-25 and Arg-211, but not Asp-59, are involved in the direct binding of PEP and GDP.

Fructose 6-phosphate-1-kinase (EC.2.7.1.11) catalyzes the phosphorylation of ß-fructose 6-phosphate to ß-fructose 1,6-bisphosphate. The activity of this enzyme is regulated by many physiological effectors depending on its source (1-5). Bacillus stearothermophilus PFK (BsPFK), with subunit molecular weight of 34,000, is activated by ADP or GDP and inhibited by phosphoenolpyruvate (PEP). These modulators bind to the same site (4,6). The amino acid residues involved in binding the substrates and certain

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Abbreviations: PFK, fructose 6-phosphate-1-kinase; BsPFK, PFK from Bacillus stearothermophilus; PEP, phosphoenolpyruvate; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; YT, yeast extract-tryptone broth; Tris-Cl, Tris-(hydroxymethyl)aminomethane chloride; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Fru 6-P, fructose 6-phosphate; Km, Michaelis-Menten constant; K_{PEP}, dissociation constant of PEP.

0006-291X/88 $1.50

modulators have been identified from the crystallographic structure of BsPFK (6). We report here the results of mutation of some of these proposed residues to prove unequivocally the ones involved in the binding of the modulators. Our results also indicate that certain mutations at the regulatory site seem to change the allosteric characteristics of BsPFK in the absence of an effector. We have reported recently the use of this approach to study the fructose 6-phosphate binding residues at the active site of BsPFK (7).

EXPERIMENTAL PROCEDURES

Enzymes and Chemicals. Enzymes for subcloning and in vitro mutagenesis were obtained from BRL, Bethesda, MD, and New England Biolabs, Inc., Beverly, MA. Other enzymes and chemicals were purchased from Sigma Chemical Co. [α-^{35}S]thio-dATP was obtained from New England Nuclear, Inc. Bacillus stearothermophilus NCA 1503 (ATCC 7954) was obtained from the American Type Culture Collection (Rockville, MD). Oligodeoxynucleotides used for site-directed mutation were synthesized by solid support phosphite chemistry in an automated DNA synthesizer (Applied Biosystems Model 380A).

Site-Directed Mutagenesis. BsPFK gene cloned in λgt10 vector (8) was double-digested with EcoRI and ClaI. The isolated 2.5 kb Bspfk fragment was subcloned into the EcoRI-AccI sites of M13mp18. The ligation product was used to transform DH5α competent cells (BRL, Bethesda, MD) with E. coli JM109 as lawn cells. The recombinant M13/Bspfk phage was used to prepare the uracil-containing single-stranded template for the oligodeoxynucleotide-directed in vitro mutagenesis as described by Kunkel (9). M13 infection was done at 37°C for not more than 5.5 hrs to prevent any undesirable deletion of the insert. Mutants were identified either by screening plaques with ^{32}P-labeled mutagenic oligodeoxynucleotides as hybridization probes or by sequencing representative recombinants. Each entire mutated BsPFK gene was sequenced using a "Sequenase Kit" (USBC, Cleveland, OH) to check that no other mutations had occured. The 2.5 kb fragment containing the mutated BsPFK gene was subcloned into the EcoRI-HindIII sites of pBR322 and expressed in the PFK-deficient E. coli DF1020 as reported for the wild type BsPFK (10).

Purification of Cloned BsPFK. The transformed DF1020 host was grown to stationary phase in 2X YT broth (16 g tryptone, 10 g yeast extract, and 5 g NaCl per liter pH 7.5) containing 100 ug/ml ampicillin at 37°C. Cells were pelleted by centrifugation at 4°C, 5,000 Xg for 10 min, resuspended at 10 % and sonicated for 5 min at 0°C in sonication buffer (50 mM Tris-Cl, pH 7.4, 1 mM DTT, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). The crude lysate was centrifuged for 1 hr at 12,000 Xg, 4°C. The clear supernatant solution was heated at 70°C for 15 min, cooled on ice and recentrifuged. The wild type enzyme and the mutants were purified at 4°C using Cibacron Blue 3GA-Agarose Type 3000-CL-L (Sigma Chemical Co.) equilibrated with sonication buffer containing 0.1 M NaCl. The enzyme samples were made to 0.1 M NaCl, loaded on the

column, washed with at least two bed volumes of equilibration buffer, and eluted using a 0.1 M - 1.5 M NaCl gradient. BsPFK which eluted at approximately 1.1 M NaCl was desalted by dialysis against sonication buffer and concentrated by dialysis against 50 % glycerol in same buffer. The concentrated enzyme solution was stored at -20°C. Prior to enzyme assay it was dialyzed against 0.1 M Tris-Cl, pH 7.4, 1 mM DTT. The final enzyme preparations appeared pure as shown by SDS-PAGE (11) stained with Coomassie Blue. Protein concentrations were determined using the BIO-RAD Protein Assay reagent (BIO-RAD, Richmond, CA).

Enzyme Assay. BsPFK activity was measured by an assay coupled to NADH oxidation (12,13). One ml of reaction mixture contained 0.1 M Tris-Cl (pH 8.2), 10 mM $MgCl_2$ 5 mM NH_4Cl, 0.2 mM $NADH_2$, 100 ug aldolase (Type IV), 18 ug triose-phosphate isomerase and 2.4 ug glycerol 3-phosphate dehydrogenase (Type III). ATP and Fru 6-P concentrations were as indicated under Results and Discussion section. To prevent ADP-mediated effects, ATP was regenerated with 1 mM creatine phosphate and 10 ug/ml creatine phosphokinase. This regenerating system was omitted whenever the effects of ADP or GDP were studied. The NADH oxidation was monitored at 340 nm using Gilford Response I spectrophotometer at 29°C.

Determination of Kinetic Parameters. Km and Hill coefficients were calculated using the Enzfitter Program by Leatherbarrow (14) on a personal computer. The dissociation constant for PEP was calculated according to Blangy et al. (15).

RESULTS AND DISCUSSION

Site-directed mutation, expression and purification. Figure 1 shows the nucleotide sequences for the four mutants. We designate Arg-25 → Ala as RA25, Arg-211 → Ala as RA211, Asp-59 → Ala as DA59 and Asp-59 → Met as DM59. Similar to the wild type, these four mutants are all stable at 70°C for 15 min. Increased purity can be obtained by longer heating in the presence of Fru 6-P. Fru 6-P, but not Mg-ATP, renders BsPFK more heat stable. The four mutants have the same mobility in SDS-PAGE as the wild type BsPFK.

Kinetic comparison of the wild type and mutants. The wild type BsPFK and the mutants, except RA25, follow hyperbolic Michaelis-Menten kinetics with respect to ATP and Fru 6-P and exhibit the same affinity for these substrates. RA25 has the same affinity for ATP as the other mutants but has half the affinity for Fru 6-P (Table 1). It exhibits sigmoidal kinetics with respect to Fru 6-P concentrations (Figure 2) with a Hill number of 2.0.

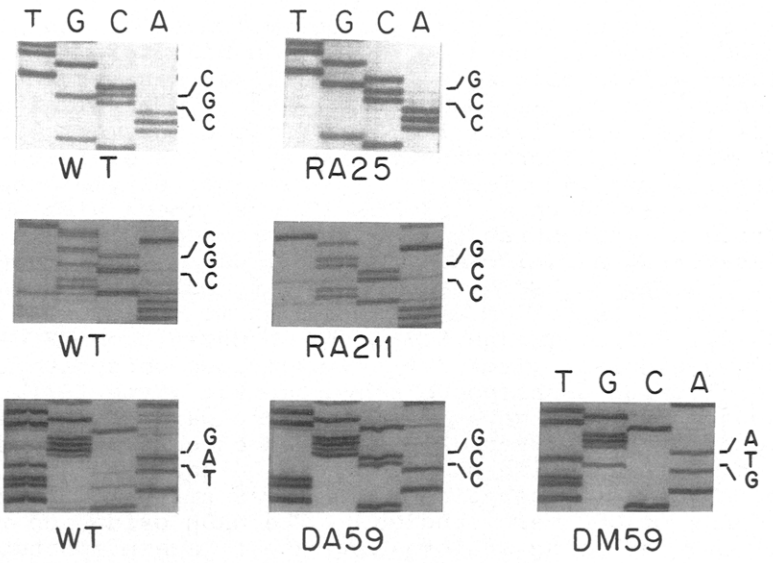

Figure 1. DNA sequence showing the mutations of Arg-25, Arg-211 and Asp-59. *In vitro* mutation was carried out according to Kunkel's protocol (9). Sequencing was performed by dideoxy chain termination method as described under the Experimental Procedures. WT, wild type; RA25, Arg-25 → Ala; RA211, Arg-211 → Ala; DA59, Asp-59 → Ala; DM59, Asp-59 → Met.

Previous kinetic studies in our laboratory have shown that the wild type BsPFK is allosterically different from the major *E. coli* PFK (7). The former exhibits a hyperbolic curve with respect to Fru 6-P whereas the latter shows sigmoidal behavior. Our data on RA25 illustrate the dramatic change in the degree of cooperativity between the subunits of BsPFK by mutating a single residue at the regulatory site, namely Arg-25 → Ala.

Effects of PEP and GDP. PEP is a potent inhibitor of the wild type BsPFK. Mutation of Asp-59 to alanine or methionine

Table 1. Kinetic parameters of the mutants and wild type BsPFK

Enzyme form	Km, Fru 6-P (mM)	K_{PEP} (mM)
Wild Type	0.029 ± 0.002	0.44 ± 0.02
RA25	0.06 ± 0.01	45 ± 9
RA211	0.029 ± 0.003	50 ± 10
DA59	0.027 ± 0.003	0.42 ± 0.07
DM59	0.028 ± 0.003	0.16 ± 0.02

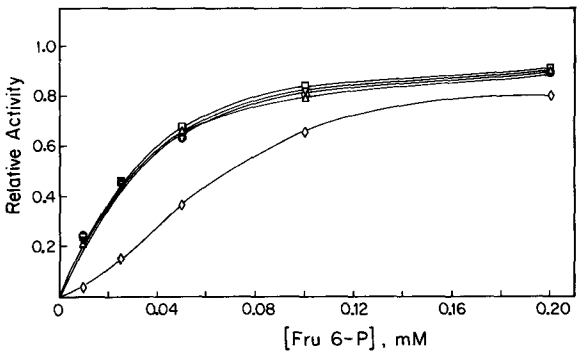

Figure 2. Comparison of the effect of Fru 6-P concentration on the activity of the wild type BsPFK and the four mutants. Activities were measured as described under the Experimental Procedures using 1 mM ATP and the indicated concentrations of Fru 6-P. ○—○, wild type; ◇—◇, RA25; ▽—▽, RA211; □—□, DA59; △—△, DM59.

renders BsPFK slightly more sensitive to PEP inhibition. The dissociation constants of PEP are 0.44, 0.42 and 0.16 mM for wild type, DA59 and DM59, respectively (Table 1). GDP reverses the inhibition by PEP (Figure 3A). These results suggest that the side chain of Asp-59 is not directly involved in the binding of PEP and GDP. This is consistent with the X-ray structure of BsPFK which shows that the polypeptide part of Asp-59, but not its side chain, binds to the β-phosphate of ADP or GDP (6).

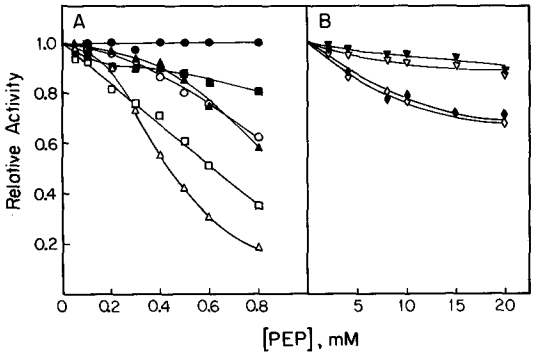

Figure 3. Effect of PEP concentrations on the activity of BsPFK. Activities were measured as described under the Experimental procedures in the presence of 1 mM ATP, 1 mM Fru 6-P and indicated concentrations of PEP in the absence (open marks) or presence (closed marks) of 0.5 mM GDP. (A) Wild Type, with (●—●) and without (○—○) GDP; DA59, with (■—■) and without (□—□) GDP; DM59, with (▲—▲) and without (△—△) GDP. (B) RA25, with (◆—◆) and without (◇—◇) GDP; RA211, with (▼—▼) and without (▽—▽) GDP.

RA25 and RA211 exhibit 100-fold less sensitivity to PEP than does the wild type (Table 1). This insensitivity to PEP inhibition and the inability of GDP to reverse this effect (Figure 3B) prove unequivocally that Arg-25 and Arg-211 are involved in the binding of both modulators. This is consistent with the crystallographic structure of BsPFK which shows that Arg-25 binds to the α- and β-phosphates of ADP or GDP and Arg-211 binds to the β-phosphate and adenine group of ADP or GDP (6).

The above-mentioned mutations define the role of Arg-25, Arg-211 and Asp-59 in BsPFK. Currently, we are conducting certain other mutations to gain more information on the mechanism of regulation of BsPFK and the interaction between its subunits.

ACKNOWLEDGMENTS: We thank M. Shirley for synthesizing the oligonucleotides. This work was supported by the National Institutes of Health Grant # DK31676.

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