Site-Directed Mutagenesis of Lysine[382], the Activator-Binding Site, of ADP-Glucose Pyrophosphorylase from *Anabaena* PCC 7120[†]

Jun Sheng, Yee-yung Charng,[‡] and Jack Preiss*

Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824

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ABSTRACT: Previous studies have shown that a highly conserved lysyl residue (Lys[419]) near the C-terminus of *Anabaena* ADP-glucose pyrophosphorylase is involved in the binding of 3-P-glycerate, the allosteric activator [Charng, Y., Iglesias, A. A., & Preiss, J. (1994) *J. Biol. Chem. 269*, 24107−24113]. Phosphopyridoxylation of the K419R mutant enzyme modified another conserved lysyl residue (Lys[382]), suggesting that this residue might be also located within the activator-binding site [Charng, Y., Iglesias, A. A., & Preiss, J. (1994) *J. Biol. Chem. 269*, 24107−24113]. Site-directed mutagenesis of Lys[382] of the *Anabaena* enzyme was performed to determine the role of this residue. Replacing Lys[382] with either arginine, alanine, or glutamine produced mutant enzymes with apparent affinities for 3-P-glycerate 10−160-fold lower than that of the wild-type enzyme. The glutamic acid mutant enzyme was inhibited by 3-P-glycerate. These mutations had lesser impact on the kinetic constants for the substrates and inhibitor, P_i, and on the thermal stability. These results indicate that both the charge and size of the residue at position 382 influence the binding of 3-P-glycerate. Site-directed mutagenesis was also performed to obtain a K382R-K419R double mutant. The apparent affinity for 3-P-glycerate of this double-mutant enzyme was 104-fold lower than that of the wild-type enzyme, and the specificity for activator of this mutant enzyme was altered. The K382R-K419R enzyme could not be phosphopyridoxylated, suggesting that other lysine residues are not involved in the binding of 3-P-glycerate.

ADP-glucose pyrophosphorylase (ATP:α-D-glucose-1-phosphate adenylyltransferase, EC 2.7.7.27) catalyzes the conversion of glucose-1-P and ATP to ADP-glucose and pyrophosphate (PP_i).[1] This allosterically regulated enzyme catalyzes the first committed step in the biosynthesis of glycogen in bacteria (Preiss & Romeo, 1989) and starch in plants (Preiss, 1991). ADP-glucose pyrophosphorylase from higher plants is mainly activated by 3-P-glycerate (3PGA) and inhibited by orthophosphate (P_i) (Ghosh & Preiss, 1965; Sanwal et al., 1968), whereas the enzyme from enteric bacteria is activated by fructose-1,6-P_2 (FBP) and inhibited by AMP (Preiss et al., 1966). The bacterial enzyme is homotetrameric in structure (Haugen et al., 1976), while the higher-plant enzyme is more complex, being heterotetrameric with two different subunits (Preiss, 1991).

Cyanobacteria are prokaryotic organisms that have metabolic properties similar to chloroplasts of higher plants (Aitken, 1988). However, the former organisms synthesize glycogen as the major carbohydrate reserve (Shively, 1988), in a similar manner to what is observed in bacteria. The cyanobacterial ADP-glucose pyrophosphorylase is homotetrameric similar to the *Escherichia coli* (*E. coli*) enzyme, but is regulated by 3PGA and P_i like the higher-plant enzyme (Levi & Preiss, 1976; Iglesias et al., 1991). Analysis of the deduced amino acid sequence indicated that the cyanobacterial enzyme is more similar to the higher-plant enzyme than to the enteric bacterial enzyme in primary structure (Smith-White & Preiss, 1992). It is of great interest to understand the structure−function relationships of the allosteric site of the cyanobacterial ADP-glucose pyrophosphorylase due to the properties of the cyanobacterial enzyme and the key position of these photosynthetic prokaryotes during evolution (Aitken, 1988).

Previous studies have shown that the activator-binding site of the *E. coli* enzyme, Lys[39], is near the N-terminus (Parsons & Preiss, 1978; Gardiol & Preiss, 1990), while that of the spinach leaf enzyme, Lys[440], is situated toward the C-terminus (Morell et al., 1988; Ball & Preiss, 1994). Chemical modification and site-directed mutagenesis studies on the cyanobacterial ADP-glucose pyrophosphorylase from *Anabaena* have shown that Lys[419], corresponding to Lys[440] in spinach leaf enzyme, is involved in binding of the activator, 3PGA (Charng et al., 1994). Lys[419] was labeled by pyridoxal-P (PLP), an analogue of the activator, 3PGA. Replacing Lys[419] with either arginine, alanine, glutamine, or glutamic acid produced mutant enzymes with apparent affinities for 3PGA 25-, 50-, 140-, or 150-fold lower than that of wild-type enzyme, respectively. These mutant enzymes still could be activated by 3PGA, which suggested that other residue(s) might be involved in the binding of 3PGA. Chemical modification studies of the K419R enzyme have shown that PLP can also be incorporated into this mutant enzyme. The phosphopyridoxylated K419R enzyme is less dependent on the presence of 3PGA for activation and less sensitive to P_i inhibition. Lys[382] of the mutant

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* To whom correspondence should be addressed. Telephone: 517-353-3137. Fax: 517-353-9334.

[‡] Present address: Mann Laboratory, Department of Vegetable Crops, University of California, Davis, CA 95616.

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[1] Abbreviations: PP_i, pyrophosphate; 3PGA, 3-P-glycerate; P_i, orthophosphate; FBP, fructose-1,6-P_2; PLP, pyridoxal-P; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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enzyme was found to be labeled by PLP. Kinetic and protection studies suggested that Lys[382] was also involved in binding of the allosteric activator (Charng et al., 1994).

Here, the results of further site-directed mutagenesis and chemical modification of the cyanobacterial ADP-glucose pyrophosphorylase from *Anabaena* are reported. Lys[382] was replaced with either arginine, alanine, glutamine, or glutamic acid with site-directed mutagenesis to determine the role of this residue. A double-mutant enzyme, K382R-K419R, was also generated and characterized.

MATERIALS AND METHODS

Reagents. [^{32}P]PP_i was purchased from Du Pont-New England Nuclear. [^{14}C]Glucose-1-P was from ICN Pharmaceuticals Inc. [α-^{35}S]dATP and the *in vitro* mutagenesis kit were from Amersham Corp. [4-^{3}H]PLP was synthesized and purified as described previously (Morell et al., 1988) according to the method of Stock et al. (1966) and Ahrens and Kortnyk (1969). Oligonucleotides were synthesized and purified by the Macromolecular Facility at Michigan State University. All other reagents were purchased at the highest quality available.

Bacterial Strains and Media. *E. coli* strain TG1 [K12, Δ(lac-pro), supE, thi, hsdD5/F'traD36, proA$^+$B$^+$, lacI^q, lacZΔM15] was used for site-directed mutagenesis and grown in LB medium. *E. coli* mutant strain AC70R1-504, which is deficient in ADP-glucose pyrophosphorylase activity (Carlson et al., 1976), was used for expression of the *Anabaena* ADP-glucose pyrophosphorylase gene (Charng et al., 1992) and grown in enriched medium containing 1.1% K_2HPO_4, 0.85% KH_2PO_4, 0.6% yeast extract, and 0.2% glucose, pH 7.0.

Site-Directed Mutagenesis. Plasmid pAnaE3a was used for both site-directed mutagenesis and gene expression. In pAnaE3a, a 5.5 kb *Eco*RI fragment of *Anabaena* genomic DNA containing the *Anabaena* ADP-glucose pyrophosphorylase gene and its putative promoter was ligated onto the *Eco*RI site of pUC119 plasmid (Charng et al., 1992). The orientation of the gene is opposite to the *lac* promoter of pUC119 and enables TG1 cells to synthesize single-stranded DNA containing the antisense strand of the gene using helper phage M13KO7 (Sambrook et al., 1982). Site-directed mutagenesis was performed according to the method of Sayers et al. (1988) using the *in vitro* mutagenesis kit from Amersham Corp. The Lys[382] mutant enzymes with substitution of arginine, alanine, glutamine, and glutamate acid were designated as K382R, K382A, K382Q, and K382E, respectively. The double mutant enzyme, in which both Lys[382] and Lys[419] were replaced with arginine, was obtained by performing site-directed mutagenesis on the K419R mutant gene (Charng et al., 1994) and designated as K382R-K419R. The oligonucleotides used to create the mutants are shown in Figure 1. The plasmids recovered in the last step of the mutagenesis were screened by dideoxy sequencing (Sanger et al., 1977) in the regions of the desired mutations. Prior to expression of the mutant enzymes, the entire coding regions of these mutant alleles were sequenced to verify that there were no unintended mutations.

Expression and Purification of the Wild-Type and Mutant Enzymes. The wild-type and mutant genes were expressed in AC70R1-504 cells. Cell extracts were prepared by sonication. The enzymes were purified by ion-exchange chromatography on DEAE-Sepharose, FPLC chromatography on Mono Q, and phenyl-Superose columns as described previously (Iglesias et al., 1991; Charng et al., 1992).

	I	I	D	K	N	A	R
	5'- ATC	ATC	GAT	AAA	AAT	GCC	CGC -3'
	3'- TAG	TAG	CTA	TTT	TTA	CGG	GCG -5'
Arg-382	5'- ATC	ATC	GAT	CGT	AAT	GCC	CGC -3'
Ala-382	5'- ATC	ATC	GAT	GCT	AAT	GCC	CGC -3'
Gln-382	5'- ATC	ATC	GAT	CAG	AAT	GCC	CGC -3'
Glu-382	5'- ATC	ATC	GAT	GAA	AAT	GCC	CGC -3'

FIGURE 1: Nucleotide sequence and encoded protein sequence of the *Anabaena* ADP-glucose pyrophosphorylase gene in the region of Lys[382] (upper) and the synthetic oligonucleotides used for site-directed mutagenesis at position 382 (lower). The position 382 codons are underlined.

Enzyme Assay. *(A) Assay I*. Enzymatic activity was measured in the pyrophosphorylase direction at 37 °C according to Preiss et al. (1966) during enzyme purification. The reaction mixtures contained 80 mM Hepes−NaOH buffer (pH 7.0), 2 mM ADP-glucose, 8 mM $MgCl_2$, 2 mM [^{32}P]PP_i (about 1500−3000 cpm/nmol), 4 mM 3PGA, 4 mM NaF, 50 μg of bovine serum albumin, and enzyme in a total volume of 250 μL. For the K382Q and K382R-K419R enzymes, 16 mM 3PGA was included in the reaction.

(B) Assay II. Enzymatic activity in the ADP-glucose synthesis direction at 37 °C was measured according to the method of Preiss et al. (1966) to determine kinetic constants. For assay of the wild-type enzyme in the presence of activator, reaction mixtures contained 100 mM Hepes−NaOH buffer (pH 8.0), 0.5 mM [^{14}C]glucose-1-P (about 1000 cpm/nmol), 2.5 mM ATP, 10 mM $MgCl_2$, 2.5 mM 3PGA, 50 μg of bovine serum albumin, 0.15 unit of inorganic pyrophosphatase, and enzyme in a final volume of 200 μL. For assay of the mutant enzymes, the reaction conditions were identical to wild-type, except that the amount of 3PGA was altered to obtain maximal activity. For the K382R, the K382A, the K382Q, and the K382R-K419R enzymes, 5, 20, 30, and 20 mM 3PGA were used, respectively. The assay of the wild-type enzyme in the absence of activator was the same as described above except that 3PGA was omitted and the amount of ATP was 5 mM in the reaction mixture. The amounts of ATP and [^{14}C]glucose-1-P were altered for some mutant enzymes to obtain maximal activity. The amount of ATP was increased to 8 mM for K382Q and K382E enzymes. For the K382R, the K382A, and the K382R-K419R enzymes, 3 mM [^{14}C]glucose-1-P was used, while 2 mM was used for K382E.

Kinetic Characterization. Kinetic data were plotted as initial velocity *versus* substrate or effector concentration. Saturating concentrations of substrates and effectors were determined to ensure that maximal velocity was attained. Data were replotted as double-reciprocal plots to determine V_{max}. Kinetic constants from hyperbolic plots were also determined by double-reciprocal plots. Sigmoidal plots were replotted as Hill plots to obtain kinetic constants. Interaction coefficients, n_H, were also determined by Hill plots. Kinetic constants were expressed as $A_{0.5}$, $S_{0.5}$, and $I_{0.5}$, which correspond to the concentration of activator, substrate, or inhibitor giving 50% of maximal activation, velocity, and

inhibition, respectively. All the kinetic parameters calculated from double-reciprocal or Hill plots were in good agreement with those obtained by using a computer program (Canellas & Wedding, 1980) which performed nonlinear iterative least-squares fitting to the Hill equation.

Protein Assay. Protein concentration was determined by using bicinchoninic acid reagent (Smith et al., 1985) with bovine serum albumin as the standard.

Protein Electrophoresis and Immunoblotting. Polyacrylamide gel electrophoresis with SDS (SDS−PAGE) was performed according to Laemmli (1970). Following electrophoresis, proteins on the gel were visualized by staining with Coomassie Brilliant Blue R-250 or electroblotted onto a nitrocellulose membrane according to Burnette (1981). After electroblotting, nitrocellulose membranes were treated with affinity-purified anti-spinach leaf ADP-glucose pyrophosphorylase IgG (Morell et al., 1987), and the antigen−antibody complex was visualized via treatment with alkaline phosphatase linked goat anti-rabbit IgG followed by staining with BM purple AP-substrate precipitating reagent (from Boehringer Mannheim GmbH).

Molecular Mass Determination. The molecular masses of the purified wild-type and mutant enzymes were determined on sucrose density gradients according to Martin and Ames (1961). The markers used were rabbit muscle lactate dehydrogenase (MW 144 000) and rabbit muscle pyruvate kinase (MW 237 000) (Worthington, 1988).

Thermal Stability. The purified enzymes were diluted to the same concentration, 0.2 mg/mL, in 50 mM Hepes−NaOH (pH 7.0) containing 1 mg/mL bovine serum albumin. The samples were heated for 5 min in a 60 °C water bath and then immediately placed on ice. The activities of these heated enzymes were assayed in the pyrophosphorolysis direction as described above.

Reductive Phosphopyridoxylation. One hundred micrograms of the K382R-K419R enzyme in 50 mM Hepes−NaOH (pH 8.0) was incubated with 0.1 mM or 0.5 mM [^{3}H]PLP (about 38 000 cpm/nmol) in a final volume of 1 mL in the dark at room temperature for 30 min. Then 100 μL of $NaBH_4$ was added to a final concentration of 49 mM to reduce the Schiff base formed between PLP and lysine residues. The reaction mixture was incubated in the dark at room temperature for another 60 min. The incorporation of [^{3}H]PLP into protein was measured by determining trichloroacetic acid precipitable counts as described previously (Charng et al., 1994).

RESULTS

Expression and Purification of Lys^{382} Mutant Enzymes. Normal expression of the mutant enzymes was confirmed by resolving the crude extract proteins with SDS−PAGE. *Anabaena* ADP-glucose pyrophosphorylase was identified by immunoblotting with antibody prepared against the spinach leaf ADP-glucose pyrophosphorylase that has been shown to be reactive with the *Anabaena* enzyme (Iglesias et al., 1991; Charng et al., 1992). All the mutant enzymes were expressed at a level similar to the wild-type enzyme based on the result of immunoblotting. The apparent sizes of these mutant enzymes were the same as that of the wild-type enzyme. The mutant enzymes were purified to greater than 90% homogeneity as estimated by SDS−PAGE of about 5 μg of protein.

Table 1: Kinetic Parameters for Activation of the Wild-Type and Mutant ADP-Glucose Pyrophosphorylases[a]

	$A_{0.5}$(3PGA) (mM)	V_{max}(−3PGA) (units/mg)[b]	V_{max}(+3PGA) (units/mg)
wild-type	0.050 ± 0.005 (1.0)	6.9 ± 0.3	60 ± 4
K382R	0.53 ± 0.05 (1.7)	0.58 ± 0.04	61 ± 6
K382A	1.9 ± 0.2 (1.7)	8.8 ± 0.7	57 ± 5
K382Q	7.8 ± 0.5 (1.7)	0.87 ± 0.03	5.9 ± 0.3
K382E	5.9 ± 0.2 (1.2)[c]	0.22 ± 0.01	
K419R[d]	1.0 ± 0.1 (1.8)	0.8 ± 0.1	103 ± 11
K419E[d]	6.0 ± 0.8 (1.9)	0.4 ± 0.1	21 ± 3
K382R-K419R	5.2 ± 0.2 (2.1)	0.50 ± 0.01	1.9 ± 0.1

[a] Reactions were performed in the synthesis direction, assay II as described under Materials and Methods. Data represent the mean ± standard deviation of two independent experiments. The values in parentheses are the Hill interaction coefficients. [b] One unit of enzyme activity is expressed as the amount of enzyme required to form 1 μmol of ADP-glucose/min at 37 °C assayed in the synthesis direction as described under Materials and Methods. [c] The K382E enzyme is inhibited by 3PGA. Therefore, the constant is $I_{0.5}$ instead of $A_{0.5}$. [d] Cited from Charng et al. (1994).

Kinetic Characterization of Lys^{382} Mutant Enzymes. The apparent affinity for 3PGA decreased dramatically when Lys^{382} was replaced with either arginine, alanine, or glutamine (Table 1). The $A_{0.5}$ values for 3PGA of the K382R, the K382A, and the K382Q enzymes were about 10-, 40-, and 160-fold higher than that of wild-type enzyme, respectively. The interaction coefficients were changed from 1.0 for the wild-type to 1.7 for the mutant enzymes. This suggested that the binding of the activator to the mutant enzymes was cooperative. The K382E enzyme was not activated by 3PGA, but rather was inhibited with an $I_{0.5}$ value of 5.9 mM. This is in contrast with the K419E mutant enzyme (Charng et al., 1994) which is activated by 3PGA with an $A_{0.5}$ about 6.0 mM.

In the absence of activator, the V_{max} value of the K382A enzyme was slightly higher, about 1.3-fold, than that of the wild-type enzyme, while the V_{max} values of the K382R, the K382Q, and the K382E enzymes were about 8, 13, and 3% of the wild-type enzyme V_{max}, respectively. In the presence of saturating 3PGA, however, the V_{max} values of the K382R and the K382A enzymes were similar to that of wild-type, while the V_{max} value of the K382Q enzyme was about 10% of the wild-type V_{max}. The degree of activation increased from 8.7-fold for the wild-type enzyme to 105-fold for the K382R enzyme. However, those for the K382A and the K382Q enzymes were about 6.5- and 6.8-fold, respectively, similar to that of the wild-type enzyme (Table 1).

The apparent affinities for the substrates (ATP, glucose-1-P, Mg^{2+}) and the inhibitor (P_i) were all relatively less affected by the mutations at position 382 (Table 2), indicating that the conformations of these ligand-binding sites were essentially unchanged. The only significant changes observed were the 4- or 5-fold increase in the $S_{0.5}$ for ATP of the K382Q enzyme in the absence or presence of activator, the 6- and 5-fold increases in the $S_{0.5}$ for glucose-1-P of the K382R and the K382E enzymes in the absence of activator, respectively, and the 11-fold increase in the $I_{0.5}$ value for P_i of the K382E enzyme in the absence of activator (Table 2). These changes are smaller than the 10-, 40-, and 160-fold increases seen in the $A_{0.5}$ value for the arginine, alanine, and glutamine mutants, respectively. For the wild-type enzyme, 2.5 mM 3PGA increased the $I_{0.5}$ value for P_i from 55 μM to 1.0 mM. With the apparent affinity relatively unchanged

Table 2: Kinetic Parameters of the Wild-Type and Mutant ADP-Glucose Pyrophosphorylases[a]

		$S_{0.5}$ (n_H) for			
	3PGA	ATP (mM)	glucose-1-P (mM)	Mg^{2+} (mM)	$I_{0.5}$ (n_H) for P_i (mM)
wild-type	none	1.4 ± 0.1 (1.2)	0.043 ± 0.001 (0.9)	6.4 ± 0.1 (6.7)	0.055 ± 0.002 (1.8)
	2.5 mM	0.11 ± 0.01 (1.2)	0.034 ± 0.004 (0.9)	3.0 ± 0.3 (4.2)	1.00 ± 0.02 (6.0)
K382R	none	1.3 ± 0.1 (1.0)	0.26 ± 0.02 (1.0)	6.6 ± 0.1 (6.9)	0.15 ± 0.01 (1.1)
	5 mM	0.22 ± 0.01 (1.5)	0.034 ± 0.004 (1.1)	3.8 ± 0.1 (4.7)	0.40 ± 0.01 (2.6)
K382A	none	3.1 ± 0.3 (1.5)	0.10 ± 0.01 (0.5)	5.6 ± 0.1 (11.4)	0.16 ± 0.02 (1.5)
	20 mM	0.30 ± 0.01 (1.9)	0.062 ± 0.004 (1.4)	3.7 ± 0.1 (3.5)	0.38 ± 0.01 (2.6)
K382Q	none	6.1 ± 0.2 (1.9)	0.053 ± 0.005 (1.0)	7.0 ± 0.1 (5.9)	0.18 ± 0.02 (1.4)
	30 mM	0.58 ± 0.03 (1.9)	0.061 ± 0.004 (1.1)	4.1 ± 0.1 (2.7)	0.10 ± 0.01 (1.7)
K382E	none	3.8 ± 0.1 (2.5)	0.22 ± 0.01 (2.5)	6.2 ± 0.2 (4.8)	0.62 ± 0.03 (1.1)
K419R[b]	none	1.4 ± 0.1 (1.0)	0.13 ± 0.01 (1.2)	3.1 ± 0.2 (2.0)	0.17 ± 0.01 (1.5)
	10 mM	0.27 ± 0.02 (1.6)	0.044 ± 0.006 (1.1)	3.9 ± 0.3 (3.4)	0.47 ± 0.03 (3.2)
K419E[b]	none	2.2 ± 0.4 (1.2)	0.021 ± 0.001 (1.0)	4.7 ± 0.7 (5.0)	0.045 ± 0.010 (1.1)
	20 mM	0.56 ± 0.06 (1.7)	0.070 ± 0.003 (1.0)	13 ± 1 (4.4)	0.15 ± 0.01 (1.8)
K382R-K419R	none	0.91 ± 0.04 (1.0)	0.32 ± 0.01 (0.9)	4.7 ± 0.1 (7.6)	0.21 ± 0.01 (1.5)
	20 mM	0.48 ± 0.03 (2.0)	0.11 ± 0.01 (1.1)	3.7 ± 0.2 (3.6)	0.29 ± 0.01 (2.0)

[a] Reactions were performed in the synthesis direction, assay II as described under Materials and Methods. Data represent the mean ± standard deviation of two independent experiments. The values in parentheses are the Hill interaction coefficients. [b] The kinetic parameters of the K419R and the K419E enzymes are from Charng et al. (1994).

for P_i but largely decreased for 3PGA, the K382R, the K382A, and the K382Q enzymes were more sensitive to P_i inhibition by having lower $I_{0.5}$ in the presence of 2.5 mM 3PGA, the amount which was saturating for the wild-type enzyme (Table 2).

Kinetic Characterization of the K382R-K419R Enzyme. When both Lys^{382} and Lys^{419} were replaced with arginine, the $A_{0.5}$ value for 3PGA was about 100-fold higher than that of the wild-type enzyme, while those of the K382R and the K419R enzymes were 10- and 20-fold higher than that of the wild-type enzyme, respectively (Table 1). The interaction coefficient was changed from 1.0 for the wild-type to 2.1 for this double-mutant enzyme. The V_{max} value was about 7% of the wild-type V_{max} in the absence of 3PGA and about 3% of that of the wild-type enzyme in the presence of 3PGA. Thus, catalytic efficiency was substantially decreased compared to the wild-type enzyme. The mutations at residues 382 and 419 did not cause much alteration in the apparent affinities for the substrates (ATP, glucose-1-P, Mg^{2+}) and the inhibitor (P_i) (Table 2). The only significant changes observed were the 4-fold increase in the $S_{0.5}$ for ATP in the presence of 3PGA, the 7-fold increase in the $S_{0.5}$ for glucose-1-P in the absence of activator, and the 4-fold increase in the $I_{0.5}$ for P_i in the absence of activator (Table 2). The fact that the apparent affinity for 3PGA of the K382R-K419R enzyme is lower than that of either the K382R enzyme or the K419R enzyme and the increased interaction coefficient suggests high cooperativity between Lys^{382} and Lys^{419} residues in the binding of 3PGA.

Effector Specificity. Previous studies have shown that a mutant *Anabaena* enzyme, in which Lys^{419} was replaced by a glutamine, has an altered activator specificity (Charng et al., 1995). The mutant enzyme is activated more effectively by FBP than by 3PGA at lower concentrations (Charng et al., 1995). It was of interest to examine whether the specificities for the allosteric effectors of the Lys^{382} mutant enzymes had changed. Several compounds, some of which are known as the major activators or inhibitors of other bacterial enzymes (Preiss, 1991), were used to test their effects on the mutants (Table 3). Some compounds have much different effects on the mutant enzymes than on the wild-type enzyme. But 3PGA still is the major activator for the K382R, the K382A, and the K382Q mutant enzymes, while P_i still is the most effective inhibitor for all mutant enzymes. However, the K382E enzyme cannot be activated by 3PGA, nor by any other effectors used. The wild-type enzyme is activated by 3PGA more effectively than by either FBP or AMP (Figure 2A). For the K382R-K419R enzyme, FBP and AMP activate the enzyme 5.4- and 8.3-fold at 5 mM, respectively (Table 3), and more effectively than 3PGA (Figure 2B), which has only a 2.7-fold activation (Table 3). The $A_{0.5}$ values for FBP and AMP of the double-mutant enzyme are 0.35 and 2.0 mM, respectively, which are similar to those of the wild-type enzyme, 0.11 and 1.8 mM.

Molecular Masses of the Wild-Type and Mutant Enzymes. The molecular mass of the wild-type enzyme determined on a sucrose density gradient is 197 kDa. The calculated molecular mass based on the deduced amino acid sequence is 193 kDa. This result is also in reasonable agreement with the molecular mass, 225 kDa, determined by gel filtration chromatography (Iglesias et al., 1991) and verifies a tetrameric structure for ADP-glucose pyrophosphorylase from *Anabaena*. To ascertain whether the loss of enzyme activity or the change of kinetic constants was perhaps due to the instability of the tetrameric complex, each mutant was subjected to sucrose density gradient sedimentation. The sedimentation patterns for the mutant enzymes were identical to that of the wild-type enzyme with a single peak where the apparent molecular masses ranged from 196 to 201 kDa, indicating that all the mutant enzymes retain the tetrameric structure.

Thermal Stability of Lys^{382} Mutant Enzymes. After heat treatment at 60 °C for 5 min, the activity of the wild-type enzyme remained unchanged, while the K382R, the K382A, the K382Q, and the K382E enzymes retained 94, 84, 84, and 85% activity, respectively. The result indicates that Lys^{382} is not required for the stability of the enzyme. The K382R-K419R enzyme retained about 78% activity after the same heat treatment.

Reductive Phosphopyridoxylation of the K382R-K419R Mutant Enzyme. Reductive phosphopyridoxylation of the K382R-K419R enzyme was performed to determine if additional lysine residues could be labeled. The double-mutant enzyme was activated about 2-fold by PLP, which was similar to that of the wild-type enzyme (Charng et al., 1994). The $A_{0.5}$ value of 0.20 mM was about 200-fold higher

Table 3: Specificity of Allosteric Effectors of the Wild-Type and Mutant ADP-Glucose Pyrophosphorylases[a]

	ADP-glucose formed (nmol/10 min)					
effector	wild-type	K382R	K382A	K382Q	K382E	K382R-K419R
none	0.58	0.20	0.97	0.82	1.99	1.12

		relative activity					
effector	effector concn (mM)	wild-type	K382R	K382A	K382Q	K382E	K382R-K419R
none		1.0	1.0	1.0	1.0	1.0	1.0
3PGA	2	9.8					
	5	10.7	89.5	6.4	3.1	0.6	2.7
fructose-6-P	2	5.7					
	5	6.5	7.5	1.9	1.7	1.0	1.1
fructose-1,6-P_2	2	1.8					
	5	1.9	1.9	0.2	0.9	0.7	5.4
glucose-6-P	2	3.8					
	5	5.3	2.9	1.3	1.9	0.8	0.9
glucose-1,6-P_2	2	0.4					
	5	0.4	1.8	0.2	0.2	0.9	4.6
pyruvate	2	1.0					
	5	1.1	1.3	0.9	2.3	0.9	1.0
PLP	2	2.0					
	5	2.0	16.8	0.3	0.2	0.1	1.9
P-enolpyruvate	2	6.1					
	5	6.8	10.0	0.5	0.7	0.8	2.6
NADPH	0.5	0.8	1.0	1.0	0.9	0.8	1.0
	2	0.4	0.9	0.6	0.7	0.7	0.9
hexane-1,6-diol-P_2	0.5	0.6	0.5	0.2	0.5	0.5	0.7
	2	0.5	0.3	0.1	0.2	0.3	0.6
ADP	2	0.9	1.8	0.6	1.6	0.8	2.1
	5	0.5	0.3	0.3	0.4	0.2	1.2
AMP	2	2.5	4.3	0.7	2.0	0.9	5.9
	5	3.3	1.6	0.7	1.3	0.5	8.3
P_i	2	0.1	0.3	0.1	0.2	0.1	0.3

[a] Reactions were performed in the synthesis direction, assay II as described under Materials and Methods, with the presence of effectors as indicated. Data represent the average of two duplications with less than 10% deviation. The amounts of the wild-type, the K382R, the K382A, the K382Q, the K382E, and the K382R-K419R enzymes used were 0.01, 0.04, 0.01, 0.15, 0.90, and 0.24 μg, respectively.

than that of the wild-type enzyme (Charng et al., 1994). Unlike the wild-type and the K419R enzymes (Charng et al., 1994), reductive phosphopyridoxylation had no effect on either enzyme activity or 3PGA activation of the K382R-K419R enzyme. In the presence of 0.1 or 0.5 mM [^{3}H]PLP, no incorporation of PLP into the mutant enzyme was found. Thus, no other lysine residue may be involved in PLP binding.

DISCUSSION

Previous studies of *Anabaena* ADP-glucose pyrophosphorylase have shown that Lys^{419} is located within the activator-binding site (Charng et al., 1994). Another lysyl residue, Lys^{382}, was modified by PLP when Lys^{419} was replaced by arginine (Charng et al., 1994), suggesting that Lys^{382} is also part of the activator-binding site. Alignment of all the amino acid sequences of ADP-glucose pyrophosphorylase available has shown that Lys^{382} is conserved in the cyanobacterial enzymes, in the small subunit of the higher-plant enzymes, and in the large subunit of the spinach leaf, wheat leaf, and potato tuber enzymes (Smith-White & Preiss, 1992). Site-directed mutagenesis experiments have been performed to probe and verify the function of Lys^{382} of *Anabaena* ADP-glucose pyrophosphorylase. The large effects on the $A_{0.5}$ values for 3PGA when Lys^{382} was replaced by other amino acids and the inability of 3PGA to activate the glutamic acid mutant enzyme are consistent with the view that Lys^{382} is involved in 3PGA binding.

As substitutions of Lys^{382} go from basic to neutral, the apparent affinities for 3PGA decrease. When the substituted amino acid is acidic, the mutant enzyme cannot be activated by 3PGA, suggesting that 3PGA cannot bind to the 3PGA-binding site. Thus, the cationic property seems to be the most important factor in 3PGA binding at position 382. The arginine mutant enzyme has about a 10-fold lower apparent affinity for 3PGA, indicating that charge alone is insufficient for the proper binding of 3PGA. The glutamine mutant enzyme has a much lower apparent affinity for 3PGA than that of the alanine mutant enzyme though both glutamine and alanine are neutral amino acids. Therefore, the size of the amino acid may also be important due to steric interference with proper binding of the activator, 3PGA.

Interestingly, the K382E enzyme cannot be activated by 3PGA but rather is inhibited. It seemed that 3PGA bound to the activator site induces an abnormal conformational change when Lys^{382} was replaced by glutamic acid. But this is not the case at position 419. The K419E enzyme still can be activated by 3PGA for about 50-fold with an $A_{0.5}$ value of 6.0 mM (Charng et al., 1994). Somehow, the enzyme can tolerate the negative charge at position 419 for the binding of 3PGA.

All the mutant enzymes retain at least 80% activities after heat treatment. Lys^{382} is obviously not critical to the stability of the native folded state. The kinetic constants for ATP, Mg^{2+}, and glucose-1-P are relatively unaffected for the five mutants compared to the dramatic changes in $A_{0.5}$, showing the tolerance to amino acid substitution at position 382. The mutant enzymes are tetrameric in structure as is the wild-type enzyme, also indicating that Lys^{382} is not essential for maintaining the quaternary structure of the enzyme.

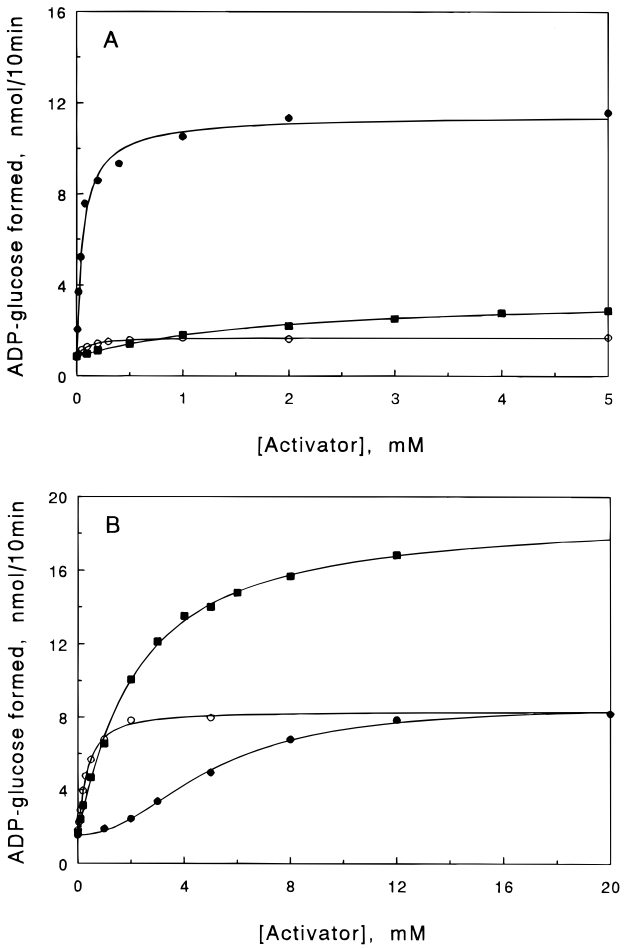

FIGURE 2: Activation of the wild-type (A) and K382R-K419R (B) enzymes by 3PGA (●), FBP (○), and AMP (■). Initial velocities of the enzymes were determined in the synthesis direction, assay II, as described under Materials and Methods with the concentration of activators being varied. The amounts of the wild-type and the K382R-K419R enzymes used were about 0.02 and 0.48 μg, respectively.

Based on previous studies (Charng et al., 1994) and our results, we can conclude that both Lys^{382} and Lys^{419} are involved in the binding of 3PGA. Both charge and size of these two lysyl residues are important for the binding of activator. However, the charge seems to be more important, and this may be explained by the interactions between the two positively charged ϵ-amino groups and the negatively charged carboxyl and phosphate groups of the activator, 3PGA. One possibility is that one of the two lysyl residues interacts with the carboxyl group and the other one with the phosphate group. The two negatively charged groups of 3PGA may interact with the two lysyl residues on the same subunit or on two different subunits. In either case, the two lysyl residues that bind to the same 3PGA molecule must be very close in three-dimensional structure.

The affinities for P_i of these mutant enzymes are relatively unaffected compared to the dramatic changes of affinities for 3PGA. This suggests that P_i has a different binding site, but the P_i binding site may overlap with the 3PGA binding site and this may explain why increasing concentrations of 3PGA reverse P_i inhibition and that P_i at higher concentrations can reverse 3PGA activation (Charng et al., 1992, 1994).

One amino acid substitution at position 382 changes the effects of almost all analogues of activator, 3PGA, on the *Anabaena* ADP-glucose pyrophosphorylase, suggesting that these analogues bind at the same site as 3PGA to activate the enzyme. Previous studies also have shown that FBP (Ghosh & Preiss, 1966; Charng et al., 1995) and P-enolpyruvate (Ghosh & Preiss, 1966) bind to the same site as 3PGA. The fact that no effectors activate the K382E enzyme can be explained by the electrostatic repulsion between anionic ligands and enzyme.

FBP and AMP become more effective as activators than 3PGA for the K382R-K419R enzyme. The alteration of the activator specificity was also observed for the K419Q *Anabaena* mutant enzyme (Charng et al., 1995). For the K419Q enzyme, FBP is about as effective as 3PGA and activates the enzyme more than 3PGA at lower concentrations. For the K382R-K419R enzyme, FBP and AMP have much higher affinities than 3PGA and activate the enzyme more than 3PGA even at higher concentrations. It should be noted that FBP and AMP are the activator and inhibitor, respectively, of the *E. coli* ADP-glucose pyrophosphorylase. Both residues in *E. coli* enzyme, corresponding to Lys^{382} and Lys^{419} in *Anabaena* enzyme, are arginine instead of lysine (Charng et al., 1992). It is suggestive that the differences of activator specificity relate to the difference of amino acids at the activator-binding site of the *E. coli* and *Anabaena*/higher-plant enzymes.

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