Arginine^{294} Is Essential for the Inhibition of *Anabaena* PCC 7120 ADP-Glucose Pyrophosphorylase by Phosphate†

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ABSTRACT: Treatment of ADP-glucose pyrophosphorylase (EC 2.7.7.27) from the cyanobacterium *Anabaena* PCC 7120 with phenylglyoxal in 50 mM Hepes, pH 8.0, at 25 °C resulted in a time- and concentration-dependent loss of enzyme activity. Phosphate, the inhibitor, protected the enzyme from inactivation most effectively, while 3-P-glycerate, fructose-1,6-P_2, pyridoxal-P, and ATP plus magnesium were also good protectors. After incubation with 2 mM phenylglyoxal for 1 h, the modified enzyme had a 10-fold lower apparent affinity for phosphate in the absence of the activator, 3-P-glycerate, than that of the wild-type enzyme. This result has implicated the involvement of an arginine residue at the allosteric sites, most probably the inhibitor-binding site, of ADP-glucose pyrophosphorylase from the cyanobacterium *Anabaena* PCC 7120. In order to identify the arginine residue, five arginine residues, which are conserved in all higher-plant and cyanobacterial enzymes but not in enteric bacterial enzymes, were individually converted to alanine by site-directed mutagenesis. The mutant enzymes, R66A, R105A, R294A, and R385A, were purified, and the properties of these mutants were compared with the wild-type enzyme. Substitution of arginine^{294} with alanine resulted in an enzyme with more than 100-fold or 40-fold lower affinity for the inhibitor, phosphate, in the absence or presence of 3-P-glycerate, respectively. This mutation had no or lesser impact on the kinetic constants for the substrates and the activator, 3-P-glycerate.

ADP-glucose pyrophosphorylase (ATP:α-D-glucose-1-phosphate adenylyltransferase, EC 2.7.7.27) is a key enzyme in the biosynthesis of glycogen in bacteria (Preiss & Romeo, 1989) and starch in plants (Preiss, 1991; Preiss & Sivak, 1996). This enzyme catalyzes the conversion of glucose-1-P and ATP to ADP-glucose and pyrophosphate (PP_i)[1] (Shen & Preiss, 1964).

ADP-glucose pyrophosphorylase is subject to allosteric regulation (Preiss & Romeo, 1989). There are significant differences between the bacterial and higher-plant enzyme. The enzyme from enteric bacteria is activated by fructose-1,6-P_2 (FBP) and inhibited by AMP (Preiss et al., 1966), whereas the enzyme from higher plants is mainly activated by 3-P-glycerate (3PGA) and inhibited by orthophosphate (P_i) (Ghosh & Preiss, 1965; Sanwal et al., 1968). In addition, the bacterial enzyme is homotetrameric in structure (Haugen et al., 1976), while the higher-plant enzyme is heterotetrameric with two different subunits (Preiss, 1991).

Cyanobacteria have metabolic properties similar to chloroplasts of higher plants (Aitken, 1988), but 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 regulated by 3PGA and P_i like the higher-plant enzyme (Levi & Preiss, 1976; Iglesias et al., 1991), but is homotetrameric similar to the *Escherichia coli* (*E. coli*) enzyme (Iglesias et al., 1991).

Previous chemical modification with pyridoxal-P and site-directed mutagenesis studies have shown that the activator-binding site of the *E. coli* ADP-glucose pyrophosphorylase, Lys^{39}, is near the N-terminus (Parsons & Preiss, 1978; Gardiol & Preiss, 1990), while those of the cyanobacterial enzyme from *Anabaena*, Lys^{382} and Lys^{419}, are close to the C-terminus (Sheng et al., 1996; Charng et al., 1994). Studies on the spinach leaf enzyme have shown that Lys^{440}, which corresponds to Lys^{419} in *Anabaena*, is involved in binding of the activator, 3PGA (Morell et al., 1988; Ball & Preiss, 1994). Studies on *E. coli*, *Synechocystis*, and spinach leaf enzymes using an arginine-specific reagent, phenylglyoxal, suggested that there is also an essential arginine residue involved in the allosteric activation and inhibition (Carlson & Preiss, 1982; Iglesias et al., 1992; Ball & Preiss, 1992).

There are five arginine residues, Arg^{66}, Arg^{105}, Arg^{171}, Arg^{294}, and Arg^{385}, conserved in the higher plant and cyanobacterial enzymes but not in the *E. coli* ADP-glucose pyrophosphorylase. Alanine scanning of the five conserved arginine reidues was therefore done to determine whether one or more of the arginine residues were important for P_i inhibition.

MATERIALS AND METHODS

Materials. Phenylglyoxal was purchased from Sigma. Stock solutions were prepared in 50 mM Hepes−NaOH (pH 8.0). [7-^{14}C]Phenylglyoxal (27.0 mCi/mmol) was from Amersham. [^{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. Oligonucleotides were synthesized and purified by the Macromolecular Facility at Michigan State University. All other reagents were purchased at the highest quality available.

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

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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.

Modification of ADP-Glucose Pyrophosphorylase with Phenylglyoxal. ADP-glucose pyrophosphorylase (0.5 μM) in 50 mM Hepes−NaOH (pH 8.0) was incubated in the dark at room temperature with different concentrations of phenylglyoxal, as indicated in the figure legends. The reactions were started by the addition of enzyme and stopped by the addition of 10 mM arginine. In protection experiments, substrates or allosteric effectors were added before the addition of enzyme. When necessary, the reaction mixtures were passed through Sephadex G-50 before assaying for activity.

Incorporation of [^{14}C]Phenylglyoxal to ADP-Glucose Pyrophosphorylase. One hundred micrograms of ADP-glucose pyrophosphorylase in 50 mM Hepes−NaOH (pH 8.0) was incubated with 4 mM [^{14}C]phenylglyoxal (11 000 cpm/nmol) in a volume of 100 μL. The incubation was carried out in the dark at room temperature. At different times, aliquots of 10 μL were removed, mixed with 2 μL of 100 mM arginine, and after 5 min put on ice. The volume of each sample was brought to 100 μL with 50 mM Hepes−NaOH (pH 8.0), and 10 μL was withdrawn to check the enzyme activity. One milliliter of cold 10% (w/v) trichloroacetic acid was added to the remaining 90 μL of sample followed by 1 h of incubation on ice. The precipitate was collected by centrifugation at 12 000 rpm for 15 min, washed twice with 1 mL of cold 10% trichloroacetic acid, and dissolved in 400 μL of 3% Na_2CO_3 in 0.1 N NaOH. Two hundred microliters of this sample was used to measure the protein concentration with a micro bicinchoninic acid reagent kit from Pierce. The radioactivity was determined by counting the other 200 μL of the sample in 5 mL of Safety Solve.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed according to the method of Sayers et al. (1988) using the *in vitro* mutagenesis kit from Amersham Corp. Plasmid pAnaE3a was used for both site-directed mutagenesis and gene expression (Charng et al., 1992, 1994; Sheng et al., 1996). The mutant enzymes with alanine substitution at positions 66, 105, 171, 294, and 385 were designated as R66A, R105A, R171A, R294A, and R385A, respectively. The oligonucleotides used for conversion of arginine to alanine are shown below:

R66A 5′- TCTCTCAATGCTCACATTGCC-3′

R105A 5′- GATGCTGTAGCTCAGTATCTC-3″

R171A 5′- AACTCTGGAGCTGTCATTGAT-3′

R294A 5′- ACCCGCGCTGCTTACTTACCA-3′

R385A 5′- AACAATGCCGCTATCGGTCAC-3′

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. 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 enzyme was purified as previously described (Iglesias et al., 1991; Charng et al., 1992) with some modification. Frozen cells were thawed into the extraction buffer containing 20 mM K-P$_i$, pH 7.5, 5 mM DTT, and 1 mM EDTA (about 5 mL of buffer/g of cells). The cell paste was sonicated for four 1 min intervals (1 min on ice between two sonications). The homogenate was centrifuged at 10 000 rpm for 10 min. The supernatant was absorbed onto a DEAE-Sepharose Fast-Flow column (about 0.03 mL bed volume/mg of protein) that had been equilibrated with 20 mM K-P$_i$ buffer, pH 7.5, containing 2 mM DTT. After the column was washed, the enzyme was eluted with a linear gradient consisting of 5 bed volumes of the above buffer in the mixing chamber and 5 bed volumes of 50 mM K-P$_i$, pH 6.0, containing 2 mM DTT and 0.3 M KCl in the reservoir chamber. Fractions of 9 mL were collected, and those containing activity were pooled. Ammonium sulfate was added into the sample with stirring to a final concentration of 50% saturation (35 g/100 mL). After centrifugation at 8000 rpm for 20 min, the pellet was put on ice for 30 min and resuspended in 1.2 M ammonium sulfate, pH 7.5 (about 0.1 mL/mg of protein). After centrifugation, the new pellet was dissolved in buffer A (about 0.5 mL/mg of protein) for Mono Q [20 mM Bis-Tris-propane, 5 mM K-P$_i$, 1 mM EDTA, 10% sucrose (w/v), and 2 mM DTT, pH 7.0] and dialyzed against the same buffer. After dialysis, the sample was applied to a Mono Q HR16/10 column equilibrated with buffer A. The column was washed with 100 mL of buffer A and eluted with two linear KCl gradients (50 mL, 0−0.1 M; 200 mL, 0.1−0.3 M) in buffer A. Fractions of 5 mL were collected, and those containing activity were pooled. The pooled sample was concentrated to a final concentration about 2 mg/mL in an Amicon concentrator fitted with a YM-30 membrane.

The R66A and the R294A enzymes were purified to homogeneity as the wild-type enzyme. The purification procedure for the R105A and the R385A enzymes was the same as for the R294A enzyme except that after the pellet was resuspended in 1.2 M ammonium sulfate, pH 7.5, and centrifuged, the new pellet was dissolved in 50 mM Hepes−NaOH, 2 mM DTT, pH 8.0 (about 0.5 mL/mg of protein).

Enzyme Assay. *(A) Assay I.* Enzymatic activity was measured in the pyrophosphorolysis 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.

(B) Assay II. Enzymatic activity was measured in the ADP-glucose synthesis direction at 37 °C according to the method of Preiss et al. (1966). (A) Activated conditions: The synthesis of ADP-[^{14}C]glucose from [^{14}C]glucose-1-P and ATP was measured in the presence of activator, 3PGA. The reaction mixture 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. (B) Unactivated conditions: The assay 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. For assay of the R66A and the R294A enzymes, the reaction conditions were identical to wild-type, except that 1.0 mM [^{14}C]glucose-1-P was used to obtain maximal activity.

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 (Hill, 1913; Atkinson et al., 1965). The expression log $(\Delta v/V_{max} - v) = n_H$ log [activator or substrate] − log $A_{0.5}$ (or $S_{0.5}$) was used for activator and substrate plots. V_{max} is the velocity of the reaction at saturating concentrations of activator or substrate, and v is the velocity of the enzyme reaction. For sigmoidal inhibition, the data were plotted according to the Hill equation (Taketa & Pogell, 1965): log $[(V_0 - v)/v] = n_H$ log [inhibitor] − log $I_{0.5}$, where V_0 is the velocity in the absence of inhibitor and v is the velocity in the presence of inhibitor. n_H, the Hill interaction coefficient, and kinetic constants, $A_{0.5}$, $S_{0.5}$, and $I_{0.5}$, which correspond to the concentration of activator, substrate, or inhibitor giving 50% of maximal activation, velocity, and inhibition, respectively, were determined from the Hill plots. 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. 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).

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.

RESULTS

Inactivation of ADP-Glucose Pyrophosphorylase by Phenylglyoxal. ADP-glucose pyrophosphorylase was inactivated by phenylglyoxal when the incubation was carried out in Hepes buffer (pH 8.0) at room temperature over a 2 h period (Figure 1). The 3PGA-activated and unactivated activities were affected by phenylglyoxal in a similar pattern. Loss of enzyme activity with time was measured at various concentrations of phenylglyoxal. The inactivations of both activated and unactivated enzyme activity followed pseudo-first-order kinetics (Figures 2A and 3A). The orders of inactivation were determined (Figures 2B and 3B) according to Levy et al. (1963). The n values were 0.95 and 0.90, respectively. This suggested that the loss of enzyme activity resulted from at least one molecule of phenylglyoxal binding per enzyme subunit.

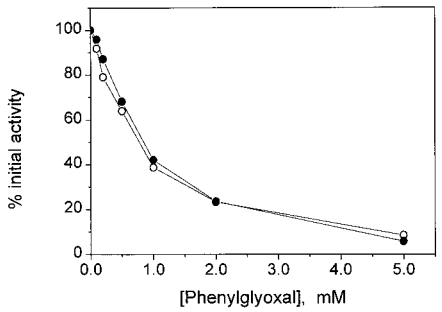

FIGURE 1: Inhibition of *Anabaena* ADP-glucose pyrophosphorylase by phenylglyoxal. Enzyme (0.5 μM) was incubated for 2 h with phenylglyoxal. Activity was measured in the presence (●) and absence (○) of 2.5 mM 3PGA (assay II), and 100% activity was 8.6 and 6.4 nmol of ADP-glucose formed per 10 min, respectively. The data represent the mean of two separate experiments.

Protection of Enzyme Activity by Substrates and Allosteric Effectors. The effects of substrates and allosteric effectors on the inactivation by phenylglyoxal are seen in Table 1. The protection against inactivation was indicated by the increase in the time required for half-inactivation. For both 3PGA-activated and unactivated activity, P_i proved to be the most effective at protecting the enzyme from inactivation by phenylglyoxal, while 3PGA and other activators, fructose 1,6-bisphosphate and pyridoxal-P, were also very good protectors. The substrate ATP plus Mg^{2+} had a similar protection against inactivation as 3PGA, while PP_i had a lesser effect. All the other substrates had no or very little protection against the inactivation by phenylglyoxal.

Effect of Modification with Phenylglyoxal on Enzyme Kinetics. Modification with phenylglyoxal made the enzyme less sensitive to P_i to inhibition (Figure 4). When the enzyme was assayed in the presence of 3PGA, the $I_{0.5}$ increased from 0.93 mM for the unmodified enzyme to 1.7 mM for the enzyme incubated with 2 mM phenylglyoxal for 1 h (Figure 4A). When the enzyme was assayed in the absence of activator, the modified enzyme had an $I_{0.5}$ about 0.27 mM, which was 10-fold higher than that of the unmodified enzyme, 0.026 mM (Figure 4B).

Inactivation of the enzyme with 1 and 2 mM phenylglyoxal for 1 h resulted in an enzyme with $A_{0.5}$ values of 0.12 and 0.22 mM, respectively, 2- and 4-fold higher than that of the unmodified enzyme, 0.058 mM.

Stoichiometry of Modification. Stoichiometry of the modification by phenylglyoxal was examined by measuring the [^{14}C]phenylglyoxal incorporation during enzyme inactivation. Figure 5 shows the incorporation of phenylglyoxal into the enzyme followed a linear correlation with

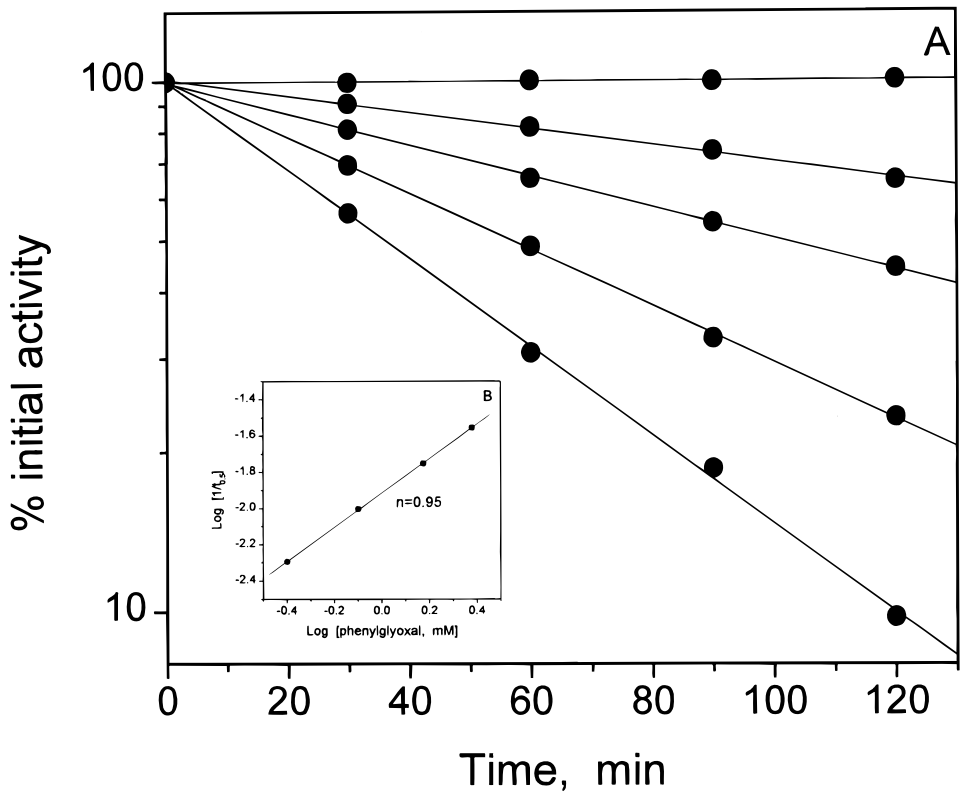

FIGURE 2: Kinetics of inhibition of ADP-glucose pyrophosphorylase activity by phenylglyoxal. (A) The loss of activity with time when enzyme (0.5 μM) was incubated with the following concentrations of phenylglyoxal: 0, 0.4, 0.8, 1.5, and 2.4 mM. The activity was measured under activated conditions (assay IIA), and 100% activity was 8.9 nmol of ADP-glucose formed per 10 min. The data represent the mean of two separate experiments. (B) Determination of the order of the reaction.

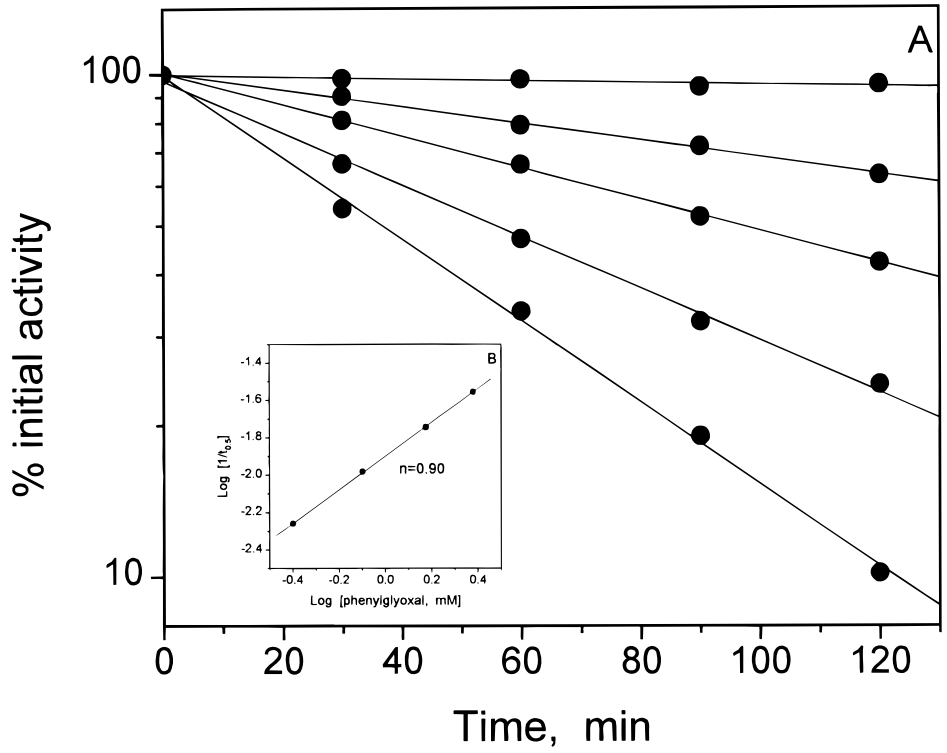

FIGURE 3: Kinetics of inhibition of ADP-glucose pyrophosphorylase activity by phenylglyoxal. (A) The loss of activity with time when enzyme (0.5 μM) was incubated with the following concentrations of phenylglyoxal: 0, 0.4, 0.8, 1.5, and 2.4 mM. The activity was measured under unactivated conditions (assay IIB), and 100% activity was 6.9 nmol of ADP-glucose formed per 10 min. The data represent the mean of two separate experiments. (B) Determination of the order of the reaction.

the loss of enzyme activity. At full inactivation, about 8 mol of phenylglyoxal was incorporated per mole of enzyme. Since phenylglyoxal reacts with arginine in a 2:1 stoichiometry (Takahashi, 1968), and the enzyme is a tetramer, this corresponds to 1 arginine residue modified per subunit.

Table 1: Effect of Substrates and Allosteric Effectors on Modification of *Anabaena* ADP-Glucose Pyrophosphorylase by Phenylglyoxal

substrate/effector	concentration (mM)	$t_{0.5}$ (min) +3PGA	$t_{0.5}$ (min) −3PGA
none		36	39
$MgCl_2$	2	41	41
ATP + 10 mM $MgCl_2$	2	240	375
ADP-glucose + 10 mM $MgCl_2$	2	85	70
glucose-1-P	2	50	60
PP_i	2	180	70
P_i	2	370	700
3-P-glycerate	2	220	300
fructose 1,6-bisphosphate	2	220	220
pyridoxal-P	0.05	210	145

[a] Enzyme was incubated with 2 mM phenylglyoxal in the presence of different ligands at the stated concentrations. Aliquots were removed at different times, and the modifications were stopped with 10 mM arginine. Activity of the modified enzyme was assayed in the presence and absence of 3PGA.

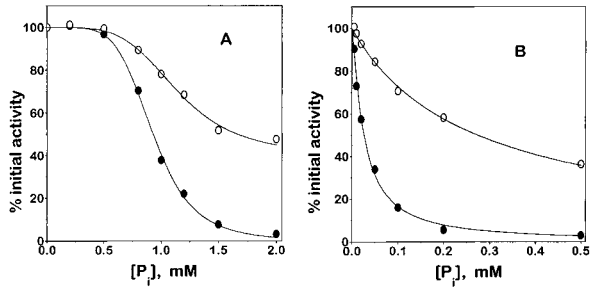

FIGURE 4: Inhibition of ADP-glucose pyrophosphorylase by P_i. The enzyme was incubated for 1 h in the absence (●) or presence (○) of 2 mM phenylglyoxal. After incubation, enzyme activity was assayed under activated (A) or unactivated (B) conditions (assay II), and 100% activity was 9.1 and 8.3 nmol of ADP-glucose formed per 10 min, respectively. The data represent the mean of two separate experiments.

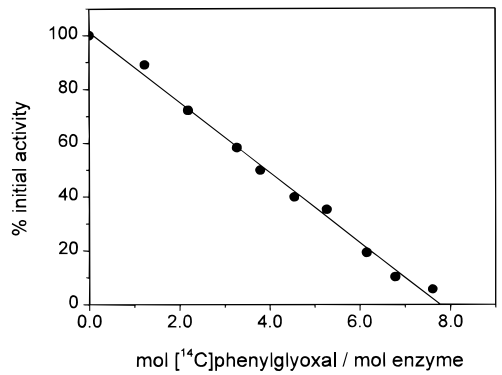

FIGURE 5: Incorporation of [^{14}C]phenylglyoxal into ADP-glucose pyrophosphorylase. Enzyme was incubated with 4 mM [^{14}C]-phenylglyoxal for different times. Aliquots were assayed for incorporated radioactivity and 3PGA activated activity (assay IIA). The data represent the mean of two separate experiments, and 100% activity was 14.3 nmol of ADP-glucose formed per 10 min.

Expression of the Mutant Enzymes. The expression of the mutant enzyme 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). The R66A, the R105A, and the R294A enzymes were expressed at a level similar to the wild-type enzyme, while the R385A enzyme was expressed less than the wild-type enzyme based on the result of immunoblotting. The apparent sizes of the mutant enzymes were the same as that of the wild-type enzyme. The R171A enzyme could not be identified by immunoblotting, and the activity in the crude extract could not be detected, while the amount of the expression plasmid in the cells was similar to that of the wild-type enzyme.

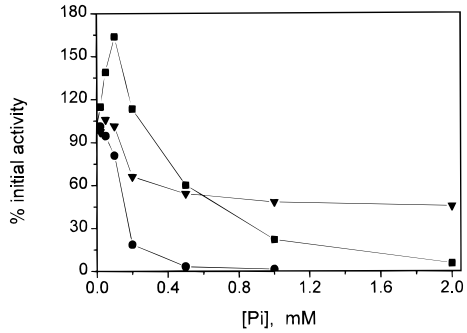

FIGURE 6: P_i inhibition of the wild-type (●), the R66A (■), and the R294A (▼) enzymes in the crude extract. Enzyme activity was assayed in the pyrophosphorolysis direction (assay I), and 100% activity was 20.6, 7.7, and 5.9 nmol of ATP formed per 10 min, respectively. The data represent the mean of two separate experiments.

Screen for Alanine-Substituted Mutant Enzymes with Altered P_i Inhibition. Enzymatic activity in the crude extract was measured in the pyrophosphorolysis direction (assay I). The R66A and the R294A enzymes were less sensitive to P_i inhibition than the wild-type enzyme (Figure 6). The inhibition of the R105A enzyme and the R385A enzyme by P_i was similar to that of the wild-type enzyme (data not shown).

Purification of the Mutant Enzymes. The R66A and the R294A enzymes were purified to greater than 90% homogeneity, while the R105A and the R385A enzymes were purified to greater than 50% and 80%, respectively, as estimated by SDS−PAGE with about 5 μg of protein.

Kinetic Characterization of the R294A Enzyme. In the synthesis direction, the apparent affinity for P_i decreased dramatically when Arg^{294} was replaced with alanine (Table 2). The $I_{0.5}$ of the wild-type enzyme was 0.055 mM, and the enzyme was inhibited 100% by 0.4 mM P_i in the absence of 3PGA, while the remaining activity of the R294A enzyme was more than 60% at 4 mM P_i (Figure 7A). In the presence of 3PGA, the $I_{0.5}$ of the wild-type enzyme was 1.0 mM, and the enzyme was 100% inhibited by 2 mM P_i, while P_i seemed to have no effect on the R294A enzyme up to 4 mM (Figure 7B).

The R294A enzyme was totally inhibited at 100 mM, and the apparent affinity for P_i was 5.2 mM or 38 mM in the absence (Figure 7A) or presence (Figure 7B) of 3PGA, respectively. The inhibition by P_i of the R294A enzyme may in large part be due to high ionic strength. Thus, KCl was tested as an inhibitor (Figure 8). KCl had very little effect on enzyme activity of the wild-type enzyme compared to P_i at lower ionic strength but inactivated the enzyme at high ionic strength (Figure 8A). The effect of KCl on R294A enzyme activity was less than P_i at low ionic strength but similar to P_i at high ionic strength (Figure 8B). This suggested that the inhibition of P_i at high concentration was mainly due to the high ionic strength.

Table 2: Kinetic Parameters of the *Anabaena* Wild-Type, R66A, R105A, R294A, and R385A Enzymes[a]

		wild-type	R66A	R105A	R294A	R385A
	3-P-glycerate			$S_{0.5}$, $I_{0.5}$, $A_{0.5}$ (n_H)		
ATP, mM	−	1.4 ± 0.1 (1.2)	1.8 ± 0.1 (1.4)	1.6 ± 0.1 (1.8)	1.2 ± 0.1 (1.5)	2.2 ± 0.1 (1.3)
	+	0.11 ± 0.01 (1.2)	0.44 ± 0.02 (1.6)	0.74 ± 0.01 (1.8)	0.13 ± 0.01 (1.3)	0.13 ± 0.01 (1.3)
glucose-1-P, mM	−	0.043 ± 0.001 (0.9)	0.094 ± 0.004 (1.0)	0.070 ± 0.008 (1.0)	0.11 ± 0.01 (0.9)	0.038 ± 0.05 (0.8)
	+	0.034 ± 0.004 (0.9)	0.042 ± 0.003 (1.0)	0.071 ± 0.007 (1.1)	0.024 ± 0.003 (1.0)	0.022 ± 0.001 (1.0)
Mg^{2+}, mM	−	6.4 ± 0.1 (6.7)	7.0 ± 0.1 (10.4)	7.3 ± 0.1 (7.4)	6.6 ± 0.2 (6.2)	6.2 ± 0.1 (7.5)
	+	3.0 ± 0.3 (4.2)	3.8 ± 0.1 (5.9)	4.5 ± 0.1 (4.1)	3.4 ± 0.1 (4.7)	3.4 ± 0.1 (4.6)
P_i, mM	−	0.055 ± 0.002 (1.8)	0.26 ± 0.01 (1.5)	0.077 ± 0.002 (1.4)	>5.2 ± 0.2 (0.6)	0.062 ± 0.002 (1.6)
	+	1.0 ± 0.02 (6.0)	0.58 ± 0.01 (3.1)	0.89 ± 0.01 (4.0)	>38 ± 1 (2.9)	0.87 ± 0.01 (5.0)
3-P-glycerate, mM		0.050 ± 0.005 (1.0)	0.087 ± 0.002 (1.4)	0.064 ± 0.02 (1.4)	0.030 ± 0.001 (1.6)	0.042 ± 0.01 (1.4)
V_{max} (unit/mg[b])	−	6.9 ± 0.3	4.8 ± 0.1	4.8 ± 0.1	11 ± 1	0.63 ± 0.01
	+	60 ± 4	44 ± 2	79 ± 2	170 ± 2	13 ± 1

[a] Data represent the mean ± standard deviation of two independent experiments. [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.

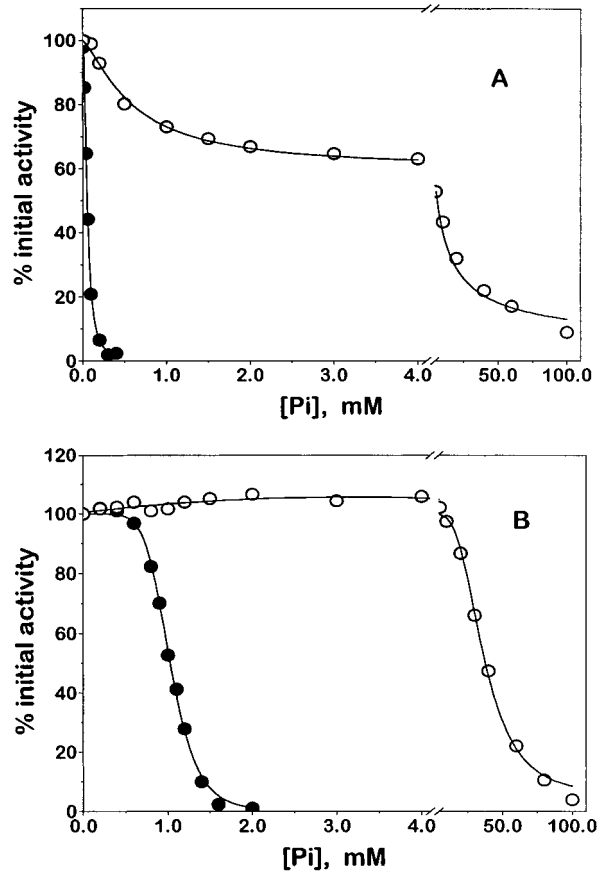

FIGURE 7: Effect of P_i on the enzyme activity of the wild-type (●) and the R294A (○) enzymes. One hundred percent activity was 6.5 and 12.0 nmol of ADP-glucose formed per 10 min, respectively, when enzyme activity was assayed in the absence (A) of 3PGA (assay IIB). When enzyme activity was assayed in the presence (B) of 3PGA (assay IIA), 100% activity was 5.4 and 11.6 nmol of ADP-glucose formed per 10 min, respectively. The data represent the mean of two separate experiments.

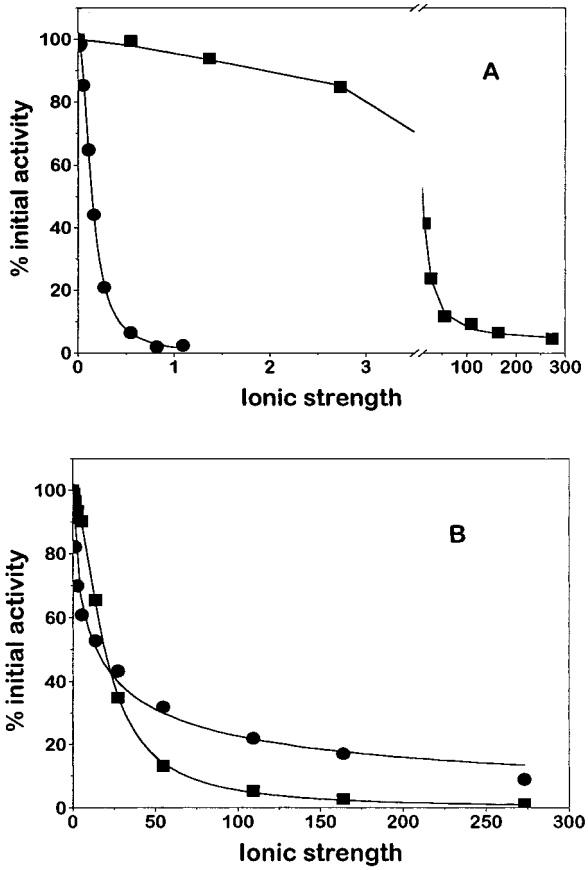

FIGURE 8: Effect of P_i (●) and KCl (■) on the enzyme activity of the wild-type (A) and the R294A enzyme (B). Enzyme activity was assayed in the absence of 3PGA (assay IIB). One hundred percent activity was 6.5 and 12.2 nmol of ADP-glucose formed per 10 min for the wild-type enzyme and 13.2 and 14.1 nmol of ADP-glucose formed per 10 min for the R294A enzyme. The data represent the mean of two separate experiments.

In the presence of saturating 3PGA, the V_{max} value of the R294A enzyme was about 3-fold higher than that of wild-type, while in the absence of the activator, the V_{max} value was about 2-fold higher. The apparent affinities for the substrates, ATP, glucose-1-P, and Mg^{2+}, and the activator, 3PGA, were all relatively unaffected by the mutation at position 294 (Table 2), indicating that the conformation of this P_i-binding site was relatively unchanged.

Kinetic Characterization of the R66A, R105A, and R385A Enzymes. The apparent affinity for P_i of the R66A enzyme was 5-fold lower than that of the wild-type enzyme, while those of the R105A and the R385A enzymes were similar to that of the wild-type (Table 2). The affinities for substrates, ATP, glucose-1-P, and Mg^{2+}, and activator, 3PGA, were all relatively unaffected by the mutations (Table 2), indicating that the conformations of these ligand-binding sites are relatively unchanged. The only significant changes observed were the 4- and 7-fold increase in the $S_{0.5}$ for ATP of the R66A and the R105A enzymes in the presence of activator and the 10- and 5-fold decrease in the V_{max} of the R385A enzyme in the absence and presence of activator, respectively (Table 2).

Thermal Stability of the Wild-Type and Mutant Enzymes. After heat treatment at 60 °C for 5 min, the activity of the wild-type and of the R66A, R105A, R294A, and R385A

enzymes retained 92, 82, 79, 88, and 75% activity, respectively. The result indicates that these arginine residues are not required for the stability of the enzyme.

DISCUSSION

Anabaena ADP-glucose pyrophosphorylase was inactivated by phenylglyoxal. At full inactivation, one arginine residue was modified per enzyme subunit. The activator 3PGA protected the enzyme from inactivation. However, the inhibitor P_i proved to be more effective at protecting the enzyme than 3PGA. In addition, substrate ATP plus Mg^{2+} also protected the enzyme from inactivation, which was not seen in previous studies with other ADP-glucose pyrophosphorylases (Carlson & Preiss, 1982; Ball & Preiss, 1992; Iglesias et al., 1992). Modification of the *Anabaena* enzyme by phenylglyoxal decreased the enzyme's sensitivity to inhibition by P_i and activation by 3PGA.

Alignment of all the amino acid sequences of ADP-glucose pyrophosphorylase available has shown that these five arginine residues, Arg^{66}, Arg^{105}, Arg^{171}, Arg^{294}, and Arg^{385}, are conserved in all the cyanobacterial and the higher-plant enzymes (Smith-White & Preiss, 1992). Considering the effect of the ionic strength on enzyme activity, the actual apparent affinity for P_i of the R294A enzyme should be more than 100- and 40-fold lower than those of the wild-type enzyme in the absence and presence of 3PGA, respectively. This large effect on the $I_{0.5}$ value for P_i when Arg^{294} was replaced by alanine suggests that Arg^{294} is involved in P_i binding.

Although the $I_{0.5}$ for P_i of the R66A enzyme in the absence of 3PGA was 5-fold higher than that of the wild-type, the change was not significant compared to the more than 100-fold change for the R294A enzyme. It was also not specific considering the $S_{0.5}$ for ATP in the presence of 3PGA and the affinity for glucose-1-P in the absence of 3PGA were changed 4- and 2-fold, respectively. Thus, Arg^{66} seems not to be directly involved in P_i inhibition.

The R105A enzyme had a 7-fold lower affinity for ATP in the presence of 3PGA while it had similar kinetic constants for other substrates, activator 3PGA, and inhibitor P_i. This suggested that Arg^{105} might be close to the ATP-binding site. It should be noted that Arg^{105} is eight amino acids away in the alignment (Smith-White & Preiss, 1992) from Tyr^{114} in *E. coli* enzyme, which is the putative ATP-binding site (Lee & Preiss, 1986).

The apparent affinities for all substrates, activator 3PGA, and inhibitor P_i of the R385A enzyme were similar to those of the wild-type enzyme. The V_{max} of this mutant enzyme was 10- or 5-fold lower than that of the wild-type enzyme in the absence or presence of 3PGA, respectively, suggesting that mutation at Arg^{385} may have resulted in conformational change and affected the enzyme's catalysis.

The R171A enzyme could not be identified by immunoblotting, and no activity could be detected in the crude extract, though the amount of the expression plasmid in the cells was similar to that of the wild-type enzyme. Arg^{171} may be important for maintaining the proper structure of ADP-glucose pyrophosphorylase. All the other mutant enzymes were expressed at a level similar to the wild-type enzyme and retained more than 75% activities after heat treatment. Arg^{66}, Arg^{105}, Arg^{294}, and Arg^{385} are obviously not critical to the stability of the native folded state. Evidence of this was also observed in the relatively unchanged kinetic constants obtained for ATP, Mg^{2+}, glucose-1-P, and 3PGA for the four mutants, showing the tolerance to amino acid substitution at these positions.

Due to the properties of the cyanobacterial ADP-glucose pyrophosphorylase and the key position of these photosynthetic prokaryotes during evolution (Aitken, 1988), it is of great interest to understand the structure−function relationships of the allosteric sites of the cyanobacterial ADP-glucose pyrophosphorylase. Previous studies have shown that Lys^{382} and Lys^{419} of *Anabaena* enzyme were involved in the binding of the activator, 3PGA. The present study clearly indicated that Arg^{294} of *Anabaena* enzyme is involved in the binding of P_i. The activator and the inhibitor obviously bind to different sites.

It would be interesting to replace Arg^{294} with another positively charged amino acid, lysine, or other neutral amino acids with larger size than alanine, leucine, or glutamine, or even a negatively charged amino acid, glutamic acid. This would show if the charge is the most important factor for binding of P_i and if the size of the amino acid at this position is also important. The fact that the corresponding amino acid at this position in the *E. coli* enzyme is glutamic acid makes it more interesting to see whether the inhibitor specificity would change or not when substituting Arg^{294} in the *Anabaena* enzyme with glutamic acid or replacing the corresponding glutamic acid in the *E. coli* enzyme with arginine. These experiments are currently in progress.

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