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Regulatory and Structural Properties of the Cyanobacterial ADPglucose Pyrophosphorylases[1]

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ABSTRACT

ADPglucose pyrophosphorylase (EC 2.7.7.27) has been purified from two cyanobacteria: the filamentous, heterocystic, *Anabaena* PCC 7120 and the unicellular *Synechocystis* PCC 6803. The purification procedure gave highly purified enzymes from both cynobacteria with specific activities of 134 (*Synechocystis*) and 111 (*Anabaena*) units per milligram protein. The purified enzymes migrated as a single protein band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis with molecular mass corresponding to 53 (*Synechocystis*) and 50 (*Anabaena*) kilodaltons. Tetrameric structures were determined for the native enzymes by analysis of gel filtrations. Kinetic and regulatory properties were characterized for the cyanobacterial ADPglucose pyrophosphorylases. Inorganic phosphate and 3-phosphoglycerate were the most potent inhibitor and activator, respectively. The *Synechocystis* enzyme was activated 126-fold by 3-phosphoglycerate, with saturation curves exhibiting sigmoidicity ($A_{0.5}$ = 0.81 millimolar; n_H = 2.0). Activation by 3-phosphoglycerate of the enzyme from *Anabaena* demonstrated hyperbolic kinetics ($A_{0.5}$ = 0.12 millimolar; n_H = 1.0), having a maximal stimulation of 17-fold. $I_{0.5}$ values of 95 and 44 micromolar were calculated for the inhibition by inorganic phosphate of the *Synechocystis* and *Anabaena* enzyme, respectively. Pyridoxal-phosphate behaved as an activator of the cyanobacterial enzyme. It activated the enzyme from *Synechocystis* nearly 10-fold with high apparent affinity ($A_{0.5}$ = 10 micromolar; n_H = 1.8). Phenylglyoxal modified the cyanobacterial enzyme by inactivating the activity in the presence of 3-phosphoglycerate. Antibody neutralization experiments showed that anti-spinach leaf (but not anti-*Escherichia coli*) ADPglucose pyrophosphorylase serum inactivated the enzyme from cyanobacteria. When the cyanobacterial enzymes were resolved on sodium dodecyl sulfate- and two-dimensional polyacrylamide gel electrophoresis and probed with Western blots, only one protein band was recognized by the anti-spinach leaf serum. The same polypeptide strongly reacted with antiserum prepared against the smaller spinach leaf 51 kilodalton subunit, whereas the anti-54 kilodalton antibody raised against the spinach subunit reacted weakly to the cyanobacterial subunit. Regulatory and immunological properties of the cyanobacterial enzyme are more related to the higher plant than the bacterial enzyme. Despite this, results suggest that the ADPglucose pyrophosphorylase from cyanobacteria is homotetrameric in structure, in contrast to the reported heterotetrameric structures of the higher plant ADPglucose pyrophosphorylase.

ADPglucose pyrophosphorylase (ATP: α-D-glucose-1-phosphate adenylyltransferase; EC 2.7.7.27) catalyzes one of the main regulatory steps in the biosynthesis of starch in plants (7, 22, 24) and glycogen in bacteria (23, 27). This enzyme mediates the synthesis of ADPglucose and PPi from ATP and glucose-1-P; the product, ADPglucose, serves as the glucosyl donor in α-glucan synthesis. All of the work done on ADPglucose pyrophosphorylase from different sources points out that there are significant differences in allosteric regulation as well as in structure between the plant and bacterial enzyme (7, 22–24, 27).

The kinetic properties of ADPglucose pyrophosphorylases from a number of plant species and tissues have been extensively studied, and in nearly all cases the enzyme is activated by 3-PGA[2] and inhibited by Pi (22, 24). Evidence accumulated from intact leaf systems supports the postulation that the [3-PGA] to [Pi] ratio regulates starch biosynthesis via modulation of ADPglucose pyrophosphorylase activity (10, 11, 18, 28). The regulatory effectors of the bacterial ADPglucose pyrophosphorylases are different, being either fructose-1,6-bisP for enteric or fructose-6-P or pyruvate for anaerobic photosynthetic bacteria (24, 27).

In *Escherichia coli* and *Salmonella typhimurium,* ADPglucose pyrophosphorylase is encoded by a single gene locus that gives rise to a homotetramer with native molecular mass of 200 kD (23). The enzyme from photosynthetic and nonphotosynthetic tissues of higher plants is more complex in structure (5, 12, 15, 16, 20, 24, 26). The purified spinach leaf enzyme possesses a molecular mass of 206 kD, which resolves into two dissimilar size subunits of 51 and 54 kD in denaturing SDS-PAGE (5, 16). These spinach leaf subunits are antigenically and amino acid sequence-wise unique, giving the native enzyme an $\alpha_2\beta_2$ heterotetrameric structure. Recently, Western blot studies indicated that dissimilar size ADPglucose pyrophosphorylase subunits are present in leaves of *Arabidopsis* (15), wheat, maize, and rice (12), as well as in maize endosperm (24, 26) and potato tuber (20). A recombinant cDNA clone for the 51 kD spinach leaf subunit has been isolated and displays significant homology to the bacterial enzyme (25). The overall biochemical and genetic evidence is in agreement with the fact that the higher plant ADPglucose pyrophosphorylase is encoded by two distinct genes (15, 16, 25).

Cyanobacteria are prokaryotic organisms with a distinctive chloroplast type of photosynthesis that bears a strong resemblance to that occurring in higher plants (1). However, the

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[2] Abbreviations: 3-PGA, 3-P-glycerate; IgG, immunoglobulin G.

former organisms synthesize glycogen as the major carbohydrate reserve (29), in a similar manner to what is observed in bacteria. The evolutionary relationship of cyanobacteria to prokaryotes and higher plants prompted a comparison of cyanobacterial ADPglucose pyrophosphorylase properties to those of *E. coli* and spinach. Previous results (14) showed that the enzyme partially purified from the blue-green bacterium *Synechococcus* 6301 is activated by 3-PGA and inhibited by Pi. In this work, we report on the purification and characterization of ADPglucose pyrophosphorylase from two cyanobacteria species: the filamentous *Anabaena* PCC 7120 and the unicellular *Synechocystis* PCC 6803.

MATERIALS AND METHODS

Cyanobacterial Strains

A glucose-tolerant strain of *Synechocystis* sp. strain PCC 6803 was provided by Dr. L. McIntosch. *Anabaena* sp. strain PCC 7120 was obtained from Dr. C. P. Wolk. Cells were grown in BG-11 medium (4) supplemented with 5 mM Tes buffer, pH 8.0. They were bubbled with air and illuminated by fluorescent light. Media to grow *Synechocystis* were further supplemented with 5 mM glucose. The cells were harvested by centrifugation, washed twice with 20 mM K-phosphate buffer, pH 7.5, containing 5 mM DTT, and stored at −80°C.

Reagents

DE-52 was obtained from Whatman. Mono Q HR 10/10 and Phenyl Superose HR 5/5 columns, molecular weight standards, and Sephacryl S-300 were from Pharmacia. BCA protein reagent was from Pierce Chemical Co. α-[^{14}C]glucose-1-P was obtained from New England Nuclear Corporation. All other reagents were of the highest quality available.

Assay of ADPglucose Pyrophosphorylase

Assay A

The pyrophosphorolysis of ADPglucose was coupled with phosphoglucomutase and glucose-6-P dehydrogenase, and NADH production followed spectrophotometrically at 340 nm at 37°C (21). Standard reaction mixtures contained in 0.5 mL: 40 μmol of Hepes-NaOH buffer (pH 7.0), 2.5 μmol of $MgCl_2$, 0.1 mg of BSA, 0.5 μmol of ADPglucose, 0.3 μmol of NAD^+, 5 nmol of glucose-1,6-bisP, 5 μmol of 3-PGA, 1 μmol of sodium pyrophosphate, and 1 unit each of rabbit muscle phosphoglucomutase and *Leuconostoc mesenteroides* glucose-6-P dehydrogenase. Assays were initiated by addition of PPi. A unit of activity is defined as the amount of enzyme catalyzing the production of 1 μmol of glucose-1-P/min under the specified conditions.

Assay B

α-[^{14}C]Glucose-1-P was used to measure the synthesis of [^{14}C]ADPglucose. Standard reaction mixtures contained in 0.2 mL: 20 μmol Hepes-NaOH buffer (pH 8.0), 50 μg of BSA, 1.5 μmol of $MgCl_2$, 0.5 μmol of ATP, 0.1 μmol of α-[^{14}C] glucose-1-P (about 1000 cpm/nmol), and 0.15 unit of inorganic pyrophosphatase. 3-PGA (0.5 μmol) was added when the enzyme was assayed in the presence of activator. Assays were initiated by addition of enzyme. Reaction mixtures were incubated for 10 min at 37°C and terminated by heating for 1 min in a boiling water bath. [^{14}C]ADPglucose was assayed as previously described (7).

Kinetic Studies

All kinetic studies were performed using assay B. $S_{0.5}$, $A_{0.5}$, and $I_{0.5}$ values, corresponding to the concentrations giving 50% maximal activity, activation, and inhibition, respectively, and Hill coefficients (n_H) were calculated from Hill plots (6). All kinetic parameters are the mean of at least two determinations and are reproducible to within at least ± 10%.

Purification of ADPglucose Pyrophosphorylase

The same purification protocol was followed for both cyanobacteria. All steps were carried out at 0 to 4°C, except when stated otherwise. Assay A was used to monitor enzyme activity throughout the purification.

Preparation of Crude Extract

Frozen cells of either *Synechocystis* 6803 or *Anabaena* 7120 were thawed into a buffer containing 20 mM K-phosphate, pH 7.5, containing 5 mM DTT (about 2 mL of buffer/g of cells). The cell paste was mixed with glass beads (about 1 g/g of cells) and then homogenized during a total time of 4 min (10 s homogenization/20 s rest cycles) in a bead beater. After washing once with additional buffer, the homogenate was centrifuged at 20,000*g* for 15 min. The pellet was washed once in additional buffer (about half the volume of the initial homogenate) and centrifuged. The combined supernatants are referred as the crude extract.

Heat Treatment

The crude extract was brought to 60°C within 5 min and kept at the same temperature for an additional 4 min. The sample was then centrifuged at 20,000*g* for 15 min at 4°C.

DEAE-Cellulose Chromatography

The supernatant from the heat treatment was absorbed onto a column of DEAE-cellulose (DE-52, about 0.1 mL bed volume/mg of protein) that had been preequilibrated with 20 mM K-phosphate buffer, pH 7.5, containing 2 mM DTT. After washing, the enzyme was eluted with a linear gradient consisting of 4-bed volumes of the above buffer in the mixing chamber and 4-bed volumes of 50 mM K-phosphate, pH 6.0, containing 2 mM DTT and 0.4 M KCl in the reservoir chamber.

Concentration

The active fractions from the DEAE-cellulose chromatography were pooled and then concentrated to 5 mL in an Amicon concentrator fitted with a PM-30 membrane.

Table I. *Purification of ADPglucose Pyrophosphorylase from Synechocystis PCC 6803*
Data correspond to a typical purification from 110 g wet cells of *Synechocystis* 6803.

Step	Vol	Total Protein	Specific Activity	Purification	Yield
	mL	*mg*	*units/mg*		%
Crude extract	400	2301	0.04	1	100
Heat treatment	380	1397	0.09	2.5	149
DEAE-cellulose	140	145.6	0.69	18.9	118
PM-30 concentration	5	69.2	1.07	29.3	87
Mono Q	25	5.25	9.19	251.6	56
Phenyl superose	3	0.61	59.4	1626	42
Sephacryl S-300	12	0.22	133.9	3666	34

Mono Q Chromatography

The concentrated sample was 10-fold diluted in a medium (buffer A) containing 20 mM Bis-Tris-propane buffer (pH 7.0), 5 mM K-phosphate, 1 mM EDTA, 10% (w/v) sucrose, and 2 mM DTT and then divided into five aliquots. These were applied to a Mono Q HR10/10 column equilibrated with buffer A. The column was washed with 40 mL buffer A and eluted with a linear KCl gradient (100 mL, 0–500 mM) in buffer A. Fractions of 5 mL were collected and those containing activity were pooled and the medium was brought to 1 M K-phosphate, pH 7.0, in order to prepare it for the next purification step.

Phenyl Superose Chromatography

The sample was absorbed onto a Phenyl Superose HR5/5 column preequilibrated with 1 M K-phosphate, pH 7.0, containing 2 mM DTT. The column was washed with the same medium until the A_{280} decreased to 0.02 and then eluted by reducing the Pi concentration in the medium through a linear gradient (20 mL, 1 M to 5 mM). Pooled peak fractions (1.5 mL each) were concentrated to 1 mL by using Centricon unit fitted with a PM-30 membrane.

Sephacryl S-300

The enzyme was chromatographed on a gel filtration column (1.5 × 96 cm) of Sephacryl S-300. The run was performed at a flow rate of about 0.14 mL/min in a medium containing 50 mM Hepes-NaOH buffer (pH 7.1), 150 mM KCl, 5 mM K-phosphate, and 2 mM DTT. Fractions of 2 mL were collected. Active fractions were pooled, concentrated with Centricon, and stored at −80°C.

Antibody Neutralization of Enzyme Activity

Neutralization of enzyme activity was tested by mixing about 0.05 unit of either *Synechocystis* 6803 or *Anabaena* 7120 ADPglucose pyrophosphorylase with antibodies raised against either spinach leaf or *E. coli* enzyme as previously described (21).

PAGE and Western Blotting

SDS-PAGE was performed in 9.5% gels according to Laemmli (13). Two-dimensional gel electrophoresis was performed as described (19), with the first dimension isoelectrofocusing developed in the pH range 3 to 10. Following electrophoresis, gels were stained for protein using silver stain or electroblotted onto nitrocellulose membranes according to Burnette (2). After electroblotting, nitrocellulose membranes were treated with affinity purified anti-spinach leaf ADPglucose pyrophosphorylase (either anti-whole enzyme or anti-51 or anti-54 kD subunits) IgG (primary antibody) and the antigen-antibody complex visualized via treatment with alkaline phosphatase linked goat anti-rabbit IgG (secondary antibody) followed by histochemical staining with 5-bromo-4-chloro-3-indoyl phosphate and nitrobluetetrazolium procedure. Immunological specificity was confirmed by performing Western blots in which the corresponding rabbit preimmune serum was substituted for affinity purified rabbit anti-ADPglucose pyrophosphorylase IgG.

Table II. *Purification of ADPglucose Pyrophosphorylase from Anabaena PCC 7120*
Data correspond to a typical purification from 40 g wet cells of *Anabaena* 7120.

Step	Vol	Total Protein	Specific Activity	Purification	Yield
	mL	*mg*	*units/mg*		%
Crude extract	180	621	0.07	1	100
Heat treatment	170	337	0.17	2.4	134
DEAE-cellulose	100	20	1.81	18.9	83
PM-30 concentration	5	15.6	2.08	29.3	75
Mono Q	5	1.23	16.41	251.6	46
Phenyl superose	6	0.21	66.0	942.9	32
Sephacryl S-300	8	0.094	110.8	1582	24

Native Molecular Mass Estimation

Molecular mass estimations were made on a column of Sephacryl S-300 (1.5 × 96 cm; 170 mL resin bed volume) using 1 mL sample volume and 40 mM Hepes-NaOH buffer (pH 7.1) containing 150 mM KCl, 5 mM K-phosphate, and 2 mM DTT as the equilibrium/elution buffer. The native molecular mass of ADPglucose pyrophosphorylase was determined from a plot of K_{AV} (partition coefficient) *versus* log molecular mass for the following proteins of known molecular weight: ferritin (440,000), catalase (232,000), aldolase (158,000), BSA (67,000).

Protein Determination

Protein concentration was measured after Smith *et al.* (30) using the Pierce Chemical Co. prepared BCA reagent and BSA as the standard.

RESULTS

Tables I and II summarize typical purification of ADPglucose pyrophosphorylase from 110 g of *Synechocystis* 6803 and 40 g of *Anabaena* 7120, respectively. As a whole, the purification followed a similar pattern for both cyanobacteria. By this procedure, *Synechocystis* 6803 ADPglucose pyrophosphorylase was purified 3666-fold with 34% yield (Table I), whereas the enzyme from *Anabaena* 7120 was purified 1582-fold with 24% recovery (Table II). To analyze these values, it is necessary to take into account that yields higher than 100% were obtained after heat treatment. This fact could be attributed to the presence of some inhibitor in the crude extract that underestimates the enzyme activity at this step. Consequently, the values of final purification and yield are overestimated. This interference was usually observed in all of the purifications made. The specific activity of the purified enzymes (134 and 111 units/mg for *Synechocystis* 6803 and *Anabaena* 7120, respectively) is very close to the values reported for highly purified ADPglucose pyrophosphorylases from bacteria (8) and higher plants (16, 24).

SDS-PAGE of the enzyme purified from each cyanobacteria revealed the presence of a single major band, indicating a purity higher than 95% by this criterion (Fig. 1). The single protein band indicated molecular masses of 53 and 50 kD for the enzyme from *Synechocystis* 6803 and *Anabaena* 7120, respectively. The molecular mass determination of the native enzyme through gel filtration chromatography in the Sephacryl S-300 column revealed values (average of three separate determinations) of 216 ± 5 kD (*Synechocystis* 6803) and 225 ± 10 kD (*Anabaena* 7120). These results indicate a tetrameric structure for ADPglucose pyrophosphorylase from cyanobacteria.

To evaluate the possible regulation of cyanobacterial ADPglucose pyrophosphorylase *in vivo,* the effect of different metabolites on the physiological activity of the enzyme was studied. Table III shows that, although with quantitative differences, the effect of the different compounds was similar for the enzyme from both cyanobacteria. In this way, 3-PGA and Pi were the most potent activator and inhibitor, respectively, of cyanobacterial ADPglucose pyrophosphorylase. Glucose-1,6-bisP, pyruvate, 2,3-bisP-glycerate, and AMP were without effect on the activity of the enzyme from both cyanobacteria (data not shown). The effect of 3-PGA seems to be highly specific, as judged by the results obtained with 2-P-glycerate, 2,3-bisP-glycerate, and glyceraldehyde-3-P (similar effect was observed with dihydroxyacetone-P). The activators of bacterial pyrophosphorylase (see refs. 24, 27) did not significantly affect the activity of the cyanobacterial enzyme. No activation by pyruvate and low activation by fructose-1,6-bisP and fructose-6-P were observed (compare with 3-PGA), indicating that the regulatory properties of the ADPglucose pyrophosphorylase from cyanobateria are more similar to the enzyme from higher plant than from bacteria (22–24). In the same way, results obtained with AMP are clearly different with the strong inhibitory effect caused by this metabolite on the enzyme from *E. coli* (22, 26).

As shown in Table III, the degree of activation by 3-PGA was significatively higher for the enzyme from *Synechocystis.* A more detailed study also revealed that the 3-PGA saturation pattern as well as the affinity for the activator were different for the enzyme from each cyanobacterium (Fig. 2). Thus, *Synechocystis* 6803 ADPglucose pyrophosphorylase exhibited a sigmoidal 3-PGA activation curve (Fig. 2A), as revealed by an upwardly curved double reciprocal plot of Δv (the difference between the velocity observed at each 3-PGA concentration and the velocity in the absence of activator, see ref. 6) against 3-PGA concentration (inset, Fig. 2A). From this plot, the maximal activation of the *Synechocystis* enzyme, reached at saturating 3-PGA concentrations, was calculated as 126-fold. From a Hill plot of the data (6), $A_{0.5}$ and n_H values of

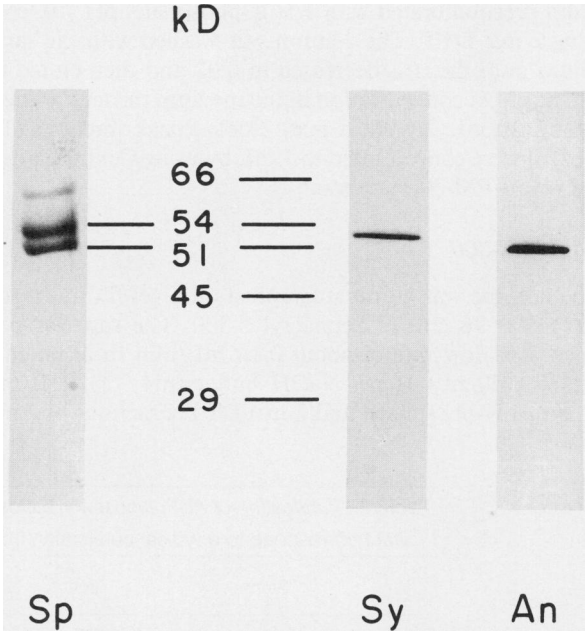

Figure 1. SDS-PAGE of ADPglucose pyrophosphorylase purified from *Synechocystis* 6803 (Sy) and *Anabaena* 7120 (An). Sp lane corresponds to a sample of spinach leaf pyrophosphorylase run in parallel. Numerals indicate molecular mass of the following standards: BSA (66 kD), spinach leaf ADPglucose pyrophosphorylase upper (54 kD) and lower (51 kD) subunit, ovoalbumin (45 kD), carbonic anhydrase (29 kD).

Table III. *Effect of Different Metabolites on the Activity of Cyanobacteria ADPglucose Pyrophosphorylase*

Enzyme activity was assayed according to assay B with the addition of the specified metabolites at a concentration of 2 mM.

Compound	*Synechocystis* 6803		*Anabaena* 7120	
	ADPglucose formed	Relative activity	ADPglucose formed	Relative activity
	nmol/10 min		*nmol/10 min*	
None	0.47	1.0	0.67	1.0
Fructose-6-P	1.53	3.3	1.92	2.9
Fructose-1,6-bisP	1.29	2.7	0.88	1.3
Glucose-6-P	1.63	3.5	1.80	2.7
P-enolpyruvate	2.97	6.3	2.26	3.4
3-P-glycerate	76.60	163.0	9.75	14.6
2-P-glycerate	0.76	1.6	1.54	2.3
Glyceraldehyde-3-P	2.05	4.4	1.33	2.0
ADP	0.51	1.1	0.66	1.0
Pi	0.08	0.2	0.13	0.2

0.81 ± 0.08 mM and 2.0 were calculated. The activation by 3-PGA for the *Anabaena* 7120 enzyme followed a hyperbolic pattern (Fig. 2B), as shown by a linear double reciprocal plot of Δv *versus* 3-PGA concentration (inset, Fig. 2B) and the n_H value of 1.0 calculated from a Hill plot of the data. The $A_{0.5}$ value for 3-PGA activation of the *Anabaena* enzyme was calculated to be 0.12 ± 0.02 mM, the maximal stimulation being 17-fold.

Concerning Pi inhibition, $I_{0.5}$ values of 95 ± 10 μM and 44 ± 4 μM were obtained for the enzyme from *Synechocystis* and *Anabaena,* respectively. For both enzymes, no cooperativity was observed for Pi inhibition (n_H near 1.0). 3-PGA affected the inhibitory effect of Pi by increasing $I_{0.5}$ values and making Pi saturation curves become sigmoidal. In the presence of 2.5 mM 3-PGA, $I_{0.5}$ values of 0.57 ± 0.06 mM and 0.46 ± 0.04 mM with n_H values of 2.2 and 1.7 were obtained for the inhibition by Pi of the enzyme from *Synechocystis* and *Anabaena,* respectively.

Table IV shows the different kinetic parameters determined for the substrates of ADPglucose pyrophosphorylase from both cyanobacteria in the absence and in the presence of 3-PGA or Pi. A comparison of the data indicates that the main difference between the enzyme from *Synechocystis* and *Anabaena* is that the former possesses a lower affinity toward ATP, with saturation curves for this substrate showing cooperativity. In the case of Mg^{2+}, although $S_{0.5}$ values were similar, a difference in the Hill number was observed. Table IV also shows that 3-PGA decreased the $S_{0.5}$ value for glucose-1-P and ATP of both enzymes and converting saturation curves hyperbolic for ATP in the *Synechocystis* protein. In the enzyme from *Synechocystis,* 3-PGA also increased both $S_{0.5}$ and n_H values for Mg^{2+}. On the other hand, Pi increased $S_{0.5}$ for glucose-1-P and ATP in both cyanobacteria enzymes and eliminated sigmoidicity in Mg^{2+} saturation curves exhibited by the enzyme from *Anabaena* (Table IV).

The activity of cyanobacterial ADPglucose pyrophosphorylase was affected by the presence of pyridoxal-P in the assay medium. Figure 3 shows a pyridoxal-P activation curve for the enzyme from *Synechocystis.* The enzyme has a higher apparent affinity for pyridoxal-P ($A_{0.5}$, 10 μM) than for 3-PGA

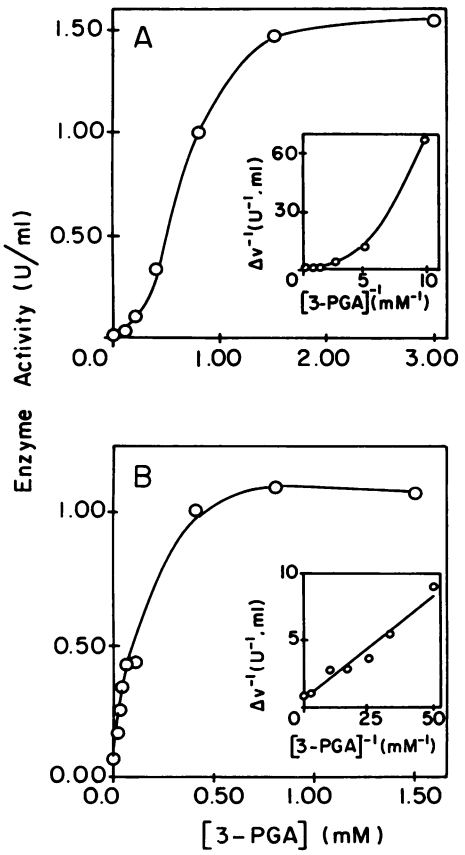

Figure 2. Activation by 3-PGA of ADPglucose pyrophosphorylase from *Synechocystis* 6803 (A) and *Anabaena* 7120 (B). The synthesis reaction mixture is described in "Materials and Methods," except that the specified amounts of 3-PGA were added. Insets: double reciprocal plot of Δv, the difference between the observed velocity at each 3-PGA concentration and the velocity in the absence of activator, against 3-PGA concentration (6).

Table IV. *Kinetic Parameters for Substrates of Cyanobacteria ADPglucose Pyrophosphorylase*

All values were determined using assay B as described in "Materials and Methods," in the absence or in the presence of 3-PGA or Pi.

Substrate	*Synechocystis* 6803		*Anabaena* 7120	
	$S_{0.5}$	n_H	$S_{0.5}$	n_H
	mM		mM	
Glucose-1-P	0.18	1.1	0.13	1.2
Glucose-1-P (+ 2.5 mM 3-PGA)	0.05	1.1	0.08	1.0
Glucose-1-P (+ 50 μM Pi)	0.46	1.1	0.39	1.0
ATP	3.20	2.2	1.55	1.2
ATP (+ 2.5 mM 3-PGA)	0.80	1.0	0.46	1.1
ATP (+ 50 μM Pi)	4.35	1.4	3.66	0.9
Mg^{2+}	1.37	1.2	1.47	2.3
Mg^{2+} (+ 2.5 mM 3-PGA)	3.75	2.5	1.62	2.8
Mg^{2+} (+ 50 μM Pi)	2.38	1.2	2.30	1.1

($A_{0.5}$, 0.81 mM), but pyridoxal-P stimulates activity only about 10-fold, compared with the over 100-fold activation reached with 3-PGA. The curve of pyridoxal-P activation of the *Synechocystis* enzyme exhibited sigmoidicity (Fig. 3), with an upwardly curved reciprocal plot of Δv against pyridoxal-P concentration (n_H = 1.8) as occurred for 3-PGA stimulation. Pyridoxal-P also activated the enzyme from *Anabaena,* but to a lesser extent, with 5 μM pyridoxal-P stimulating enzyme activity about 1.8-fold.

Incubation of *Synechocystis* ADPglucose pyrophosphorylase with the arginine specific reagent phenylglyoxal resulted in an inactivation of the enzyme when assayed in the presence of 3-PGA (Fig. 4). However, catalytic activity was only slightly decreased by arginine modification under conditions at which the enzyme was assayed in the absence of 3-PGA (Fig. 4). Inactivation was dependent on the phenylglyoxal concentration and was not reversed by dialysis of the modified enzyme. Similar results were observed for the enzyme purified from *Anabaena.* These results show that pyridoxal-P and phenylglyoxal affect the allosteric activation of the cyanobacterial ADPglucose pyrophosphorylase, suggesting the involvement of lysine and arginine residues in the 3-PGA binding to the protein.

To further compare cyanobacterial ADPglucose pyrophosphorylase with the enzyme from bacteria and higher plants, we performed antibody neutralization experiments. Figure 5 shows that incubation of the enzyme from either *Synechocystis* or *Anabaena* with antibodies raised against spinach leaf ADPglucose pyrophosphorylase resulted in a loss of enzyme activity. The amount of antiserum necessary to cause 50% inhibition was about 100 and 80 μL of antiserum per unit of *Synechocystis* and *Anabaena* enzyme, respectively. In contrast, serum containing anti-ADPglucose pyrophosphorylase from *E. coli* did not significantly affect cyanobacterial enzyme activity (Fig. 5). When similar experiments were carried out by using an anti-51 kD subunit serum of the spinach leaf enzyme, only partial inhibition (between 20 and 40%) was

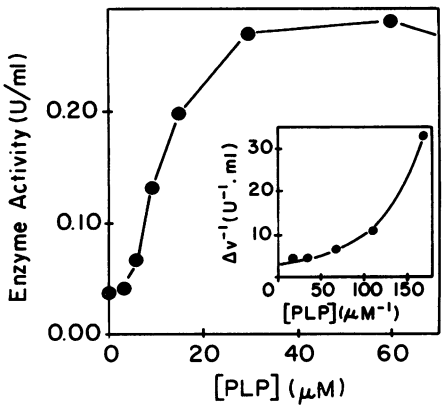

Figure 3. Activation of *Synechocystis* 6803 ADPglucose pyrophosphorylase by pyridoxal-P. The enzyme activity was determined by using assay B with the addition of pyridoxal-P at the amounts indicated. Inset: double reciprocal plot of Δv *versus* pyridoxal-P concentration.

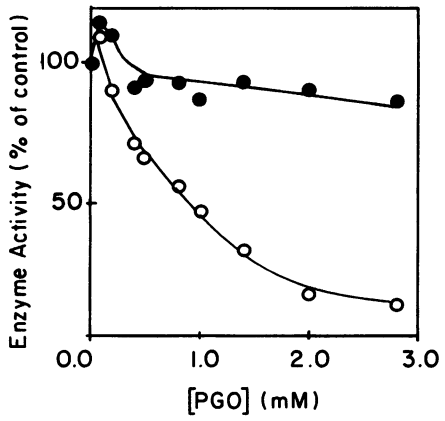

Figure 4. Modification by phenylglyoxal of *Synechocystis* 6803 ADPglucose pyrophosphorylase. Enzyme (about 0.03 mg/mL) in 40 mM Mops-NaOH, pH 8.0, was incubated at 37°C with the stated amounts of phenylglyoxal. After 1 h incubation, modification was stopped by addition of 10 mM arginine. The modified enzyme was assayed for activity using assay B in the absence (●) or in the presence (○) of 2.5 mM 3-PGA.

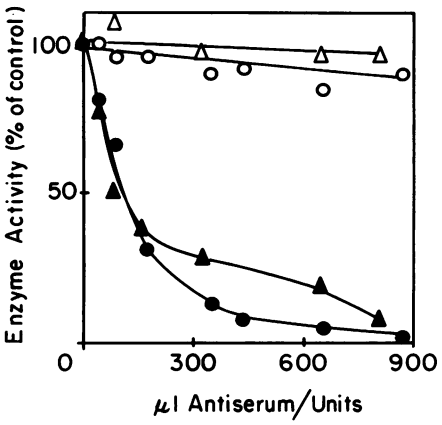

Figure 5. Neutralization of *Synechocystis* 6803 (●, ○) and *Anabaena* 7120 (▲, △) ADPglucose pyrophosphorylase by antispinach leaf (closed symbols) and anti-*E. coli* (open symbols) ADPglucose pyrophosphorylase immune serum. Assay A was used to determine activity of the enzymes from both cyanobacteria after incubation with different amounts of the corresponding antiserum.

observed, whereas anti-54 kD subunit serum had no effect at all.

The immunological relationship between cyanobacterial and spinach ADPglucose pyrophosphorylase was utilized to further characterize the enzyme from *Synechocystis* and *Anabaena* through immunoblot analysis of SDS-PAGE. Figure 6 shows Western blot hybridization performed using antibodies raised against the spinach enzyme. The anti-whole spinach leaf enzyme serum recognized only one band of ADPglucose pyrophosphorylase from either cyanobacteria (Fig. 6A). The molecular mass of this band corresponded to the band shown for the SDS-PAGE gel stained for protein (Fig. 1). The same band was strongly recognized by the anti-51 kD spinach subunit serum (Fig. 6B), whereas that the anti-54 kD serum exhibited a very weak cross-reaction (Fig. 6C). These results suggest that cyanobacterial ADPglucose pyrophosphorylase is composed of only one type of subunit. If different, such subunits are very similar (or identical) in molecular mass. The fact that Western blots from two-dimensional electrophoresis also revealed the presence of one polypeptide recognized by the anti-spinach leaf enzyme (Fig. 7) also strongly suggests a homotetrameric structure for cyanobacterial ADPglucose pyrophosphorylase.

DISCUSSION

Cyanobacteria, more than other bacteria, are closely related to higher plants, with different studies supporting the cyanobacterial origin of chloroplast (1). The intermediate position occupied by cyanobacteria during evolution makes it of interest to compare the properties of their molecular components to those from bacteria and plants. In this study, we characterized kinetic, regulatory, and structural properties of ADPglucose pyrophosphorylase, a key enzyme in the synthesis of glycogen, from two different cyanobacteria: the unicellular *Synechocystis* PCC 6803 and the filamentous *Anabaena* PCC 7120.

As shown previously in the blue-green bacterium *Synechococcus* 6301 (14), ADPglucose pyrophosphorylase from *Synechocystis* and *Anabaena* exhibited regulatory properties that resemble the higher plant enzyme. Kinetic data reported herein are, in general, in agreement with those previously reported for the *Synechococcus* enzyme (14). Of the different metabolites tested, 3-PGA and Pi were the most potent activator and inhibitor, respectively, of the enzyme from cyanobacteria, thus reinforcing the idea that the [3-PGA] to [Pi] ratio could be of importance to regulate the biosynthesis of glycogen in these organisms. The absence of inhibition by AMP and activation by pyruvate as well as the low activation caused by fructose-1,6-bisP and fructose-6-P (compared with 3-PGA stimulation) indicate that regulatory properties of cyanobacterial ADPglucose pyrophosphorylase are different from those of the bacterial enzyme (24, 27). It is interesting to note that the enzyme from *Synechocystis* showed some distinctive kinetic properties, namely (a) a higher $S_{0.5}$ value for ATP, and (b) a lower affinity toward 3-PGA, although with a very high maximal activation produced by this metabolite as compared with the *Anabaena* enzyme. Nevertheless, the activated enzyme exhibited a higher specific activity in crude extracts of *Anabaena* than in *Synechocystis*.

ADPglucose pyrophosphorylase from cyanobacteria was found to be antigenically related to the spinach leaf protein. Antibodies raised against the enzyme from spinach, but not from *E. coli*, effectively inhibited the cyanobacterial enzyme. Western blot experiments showed that only one type of sub-

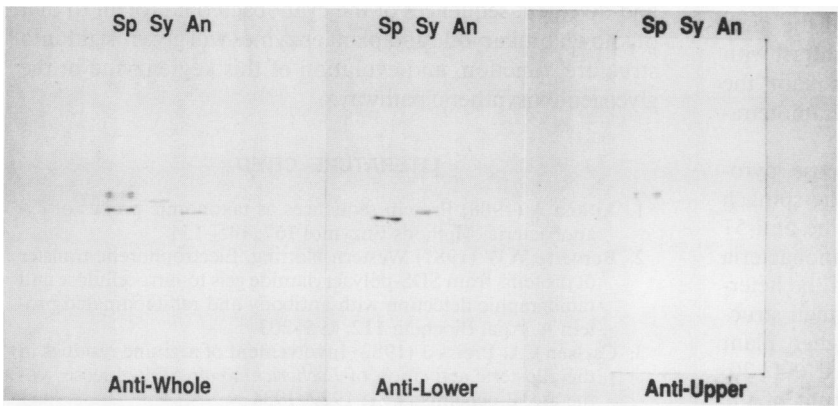

Figure 6. Western blot analysis of *Synechocystis* 6803 (Sy) and *Anabaena* 7120 (An) ADPglucose pyrophosphorylase. SDS-PAGE was transferred to nitrocellulose filters and then incubated with antisera reached against whole enzyme (Anti-Whole), 51 kD subunit (Anti-Lower), or 54 kD subunit (Anti-Upper) spinach leaf ADPglucose pyrophosphorylase. Sp lanes show samples of enzyme highly purified from spinach leaf run in parallel. The doublet observed for the 54 kD subunit of this highly purified spinach leaf preparation was attributed to a partial proteolysis of this subunit produced during storage of the sample.

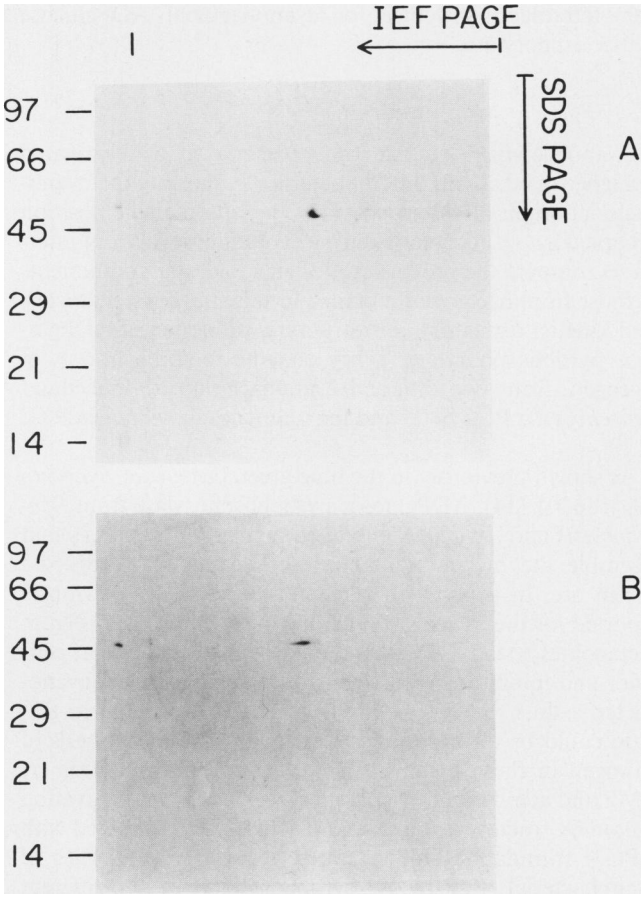

Figure 7. Analysis of purified cyanobacterial ADPglucose pyrophosphorylases by two-dimensional PAGE and Western blot analysis. The enzyme purified from *Synechocystis* 6803 (A) and *Anabaena* 7120 (B) was electrophoresed in a two-dimensional PAGE system as described by O'Farrell (19). After electrophoresis, gels were electroblotted onto nitrocellulose membranes and then probed with IgG prepared against the purified spinach leaf ADPglucose pyrophosphorylase. The directions of the denaturing isoelectric focusing (acidic to basic) and SDS-PAGE runs are indicated. Numerals correspond to prestained protein standards (from Bio-Rad) run in the SDS-PAGE second dimension: phosphorylase B (97 kD), BSA (66 kD), ovoalbumin (45 kD), soybean trypsin inhibitor (21 kD), lysozyme (14 kD).

unit could be distinguished for the cyanobacterial ADPglucose pyrophosphorylase based on its cross-reactivity to the anti-spinach serum. Thus, our results suggest that, in contrast with its close relationship with the protein from higher plant, the native enzyme from cyanobacteria seems to be homotetrameric in structure.

The cross-reaction of cyanobacterial ADPglucose pyrophosphorylase with the antibody prepared with the spinach leaf 54 kD subunit was much weaker than with the anti-51 kD subunit. This suggests that the enzyme from cyanobacteria is structurally more related to the small subunit of the heterotetrameric higher plant protein. Interestingly, a high structural homology between small subunits of higher plant ADPglucose pyrophosphoryalse has been reported (14, 20, 21, 25). The conservation of the 51 kD component of the enzyme detected in different plant tissues has been corroborated by analysis of cDNA clones. The primary sequences of the rice endosperm subunit and spinach leaf 51 kD subunit reveal about 76% identity (25). Contrarily, the larger subunit of plant ADPglucose pyrophosphorylase was found more divergent and sharing less sequence identity (20).

Pyridoxal-P behaved as allosteric activator of cyanobacterial ADPglucose pyrophosphorylase. The enzyme exhibited a higher affinity for pyridoxal-P than for the physiological activator, 3-PGA; however the maximum activation by pyridoxal-P was significantly lower compared with stimulation by 3-PGA. Pyridoxal-P has been shown to activate bacterial (9) and plant (17) ADPglucose pyrophosphorylase. Reductive phosphopyridoxylation of the *E. coli* enzyme resulted in enzyme that was fully active in the absence of the physiological activator, fructose-1,6-bisP (9). Results obtained with the enzyme from cyanobacteria are in agreement with the effect of pyridoxal-P on the spinach enzyme, in the fact that this reagent does not activate the enzyme as effectively as 3-PGA (17).

Phenylglyoxal inactivated cyanobacterial ADPglucose pyrophosphorylase when assayed in the presence of 3-PGA, without significant effect on activity in the absence of the physiological activator. These results are similar to those previously reported for the *E. coli* enzyme (3) concerning modification of arginine residues interfering with normal allosteric activation. Modification of the spinach leaf enzyme with phenylglyoxal showed some differences in the respect that, although slower, inactivation of activity in the absence of 3-PGA was also observed (K. Ball, J. Preiss, unpublished results). Thus, results obtained with the cyanobacterial enzyme suggest that phenylglyoxal primarily modifies arginine residues involved in the 3-PGA binding, as occurs with the enzyme from bacteria (3).

Results reported in this study show that ADPglucose pyrophosphorylase from cyanobacteria has structural and regulatory properties similar to the higher plant rather than bacterial enzymes (23, 24). However, the cyanobacterial protein seems to be homotetrameric in structure, as seen with the bacterial enzyme. These intermediate characteristics make the enzyme from cyanobacteria of interest, both to determine its primary structure and to compare it with the protein from other sources. In this way, pyridoxal-P and phenylglyoxal may prove useful as probes to characterize the nature and location of the allosteric binding site(s). A comparison of the conserved and divergent sequences of the cyanobacterial protein to that of known prokaryotic and plant enzymes will give insight into structure, function, and evolution of this key enzyme in the glycogen biosynthetic pathway.

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