Regulatory Properties of the ADP-Glucose Pyrophosphorylase of the Blue-Green Bacterium *Synechococcus 6301* 1

Carolyn Levi and Jack Preiss

Department of Biochemistry and Biophysics, University of California, Davis, California 95616

Received for publication July 6, 1976 and in revised form August 17, 1976

ABSTRACT

ADP-glucose was found to be the primary sugar nucleotide used for glycogen synthesis by *Synechococcus 6301*. ADP-glucose pyrophosphorylase was partially purified 12-fold from this blue-green bacterium. The enzyme was activated 8- to 25-fold by glyceraldehyde 3-phosphate. Fructose 6-phosphate, fructose 1,6-bisphosphate, 5'-adenylyltransferase, and adenosine diphosphate activated the enzyme, but less than glyceraldehyde 3-phosphate. The enzyme was inhibited by inorganic phosphate. The *f*max of phosphate was 0.072 mM, and in the presence of 2 mM glyceraldehyde 3-phosphate, increased to 1.8 mM. The substrate saturation curves for glyceraldehyde 3-phosphate and ATP were hyperbolic in both the presence and absence of glyceraldehyde 3-phosphate or phosphate. The saturation curve for MgCl2 was sigmoidal; 2 mM glyceraldehyde 3-phosphate decreased the sigmoidicity from a Hill slope *n* value of 5.6 to 2.8, and increased the MgCl2 optimum from 3 mM to 6 to 7 mM.

Previous studies have shown that ADP-glucose is the exclusive sugar nucleotide used for glycogen synthesis by many bacteria, and is the primary sugar nucleotide of starch synthesis in green algae and higher plants (7, 11, 16). α-Glucan synthesis in plants and bacteria proceeds through the synthesis of ADP-glucose from glucose-1-P and ATP by ADP-glucose pyrophosphorylase (ADP-glucose-1-P adenylyltransferase), followed by transfer of the glucosyl moiety from ADP-glucose to a glucan primer by ADP-glucose:1,4-α-d-glucan-4-α-glucosyl transferase (glucogen or starch synthase). Regulation of glycogen and starch synthesis occurs via allosteric regulation of ADP-glucose pyrophosphorylase (11, 14). ADP-glucose pyrophosphorylase activity in bacteria is regulated by metabolites of the prevalent route of carbon metabolism of the specific bacterial group (11, 14). The ADP-glucose pyrophosphorylases of green algae (18) and of higher plants (6, 19) differ from any bacterial ADP-glucose pyrophosphorylase studied thus far in that they are primarily activated by 3-P-glycerate (for reviews, see references 11 and 14).

Therefore, it was of interest to determine the mode of regulation of glycogen synthesis in blue-green bacteria. This paper reports the relative activities of the enzymes of the sugar nucleotide-dependent pathway of glycogen synthesis and the mode of regulation of the activity of partially purified ADP-glucose pyrophosphorylase from the blue-green bacterium, *Synechococcus 6301*.

MATERIALS AND METHODS

BACTERIAL STRAINS

Agar slant cultures and frozen cells of *Synechococcus 6301* (*Anacystis nidulans*) and *Aphanocapsa 6308* were gifts of R. Y. Stanier. The cultures were maintained on solid agar slants of BG-11 and grown in liquid BG-11 as shaker cultures in air (22). Cells used for enzyme purification were grown in 10- to 12-liter cultures at 35 C in a New Brunswick microfermentor. They were bubbled with air and illuminated by fluorescent lights. The cells were harvested by centrifugation, washed twice with 20 mM K-phosphate buffer, pH 7.5, and stored at -86 C.

REAGENTS

1.6-Hexanediol bisphosphate was prepared as previously described (8). Dicyclohexylamine 2-hydroxy-4-phosphonoobutyric acid was a gift of H. B. F. Dixon, University of Cambridge. Di Li 3,4-dihydroxy-buty1-phosphonate and di Li 2,3-dihydroxypropyl-1-phosphonate were gifts of B. E. Tropp, Queens College, City College of New York. Other reagents were obtained at the highest purity from commercial sources.

ASSAY PROCEDURES

Assay of ADP-Glucose Pyrophosphorylase (Assay A: Pyrophosphorylase Reaction). The reaction mixture contained 20 μmol tris-Cl, pH 7.5; 2 μmol MgCl2; 2.5 μmol NaF; 50 μg BSA; 0.4 μmol ADP-glucose; 0.5 μmol pyrophosphate; 1 μmol glyceraldehyde-3-P and enzyme in 0.3 ml total volume. The pyrophosphate formed was assayed as described by Shen and Preiss (21). This assay was used to determine enzyme activities in crude preparations and during purification. One unit of enzymic activity is defined as the amount which catalyzes the formation of 1 μmol ATP in 10 min.

Assay of ADP-Glucose Pyrophosphorylase (Assay B: Synthesis Reaction). The reaction mixture contained 20 μmol of HEPES buffer, pH 8.0; 0.24 μmol ATP; 50 μg BSA; 1 μmol MgCl2; 0.135 μmol [14C]glucose-1-P (1016 cpm/nmol or 3242 cpm/nmol); 0.33 μg crystalline yeast inorganic pyrophosphorylase; activator, and enzyme to 0.2 ml total volume. HEPES buffer, pH 7, was substituted for HEPES, pH 8, where indicated. ADP-[14C]glucose synthesized was assayed as described (6). One unit of enzymic activity is that amount which catalyzes the formation of 1 μmol ADP-glucose in 10 min.

Assay of Glycogen Synthase. The reaction mixture contained 20 μmol of Bis-Tris buffer, pH 8.5; 5 μmol K-acetate or 5 μmol KC1; 1 μmol EDTA; 2 μmol GSH; 1 mg rabbit liver glycogen; 0.48 μmol ADP-[14C]glucose (375–480 cpm/nmol) and enzyme in a total volume of 0.2 ml. [14C]Glucose incorporation into glycogen was determined by the method of Greenberg and Preiss (7). That amount of enzyme which catalyzes the incorporation of 1 μmol of glucose into glycogen in 10 min is defined as 1 unit.

Determination of Kinetic Constants. Kinetic constants were determined as described in Preiss et al. (15).

Assay of Phosphoglucomutase. The reaction mixture contained 25 μmol of tris-Cl, pH 7.5; 0.2 μg BSA; 5 μmol MgCl2; 0.065 μmol glucose-1,6-dip; 0.24 μmol NADP; 25 units glucose-6-P dehydrogenase; 4 μmol glucose-1-P; and enzyme to 1 ml total volume. Activity was followed by reduction of NADP at 340 nm.

1 Supported in part by United States Public Health Service Research Grant AI-05520.
Phycobilins were assayed by the method of Bennett and Bogorad (1).

**PREPARATION OF CRUDE CELL EXTRACT**

Crude extracts were prepared by grinding of 0.3 to 1 g frozen cells, glass beads, and 3 to 4 ml of 50 mM glycyglycine buffer, pH 7, containing 5 mM diithioerythritol in a mortar and pestle. The homogenate was centrifuged for 2 min at 2000g to remove whole cells and cell debris, and then at 10,000g for 15 min to remove small particles. The second pellet was resuspended in 0.25 to 2 ml of the homogenization buffer and used for glycyan synthase assays. The supernatant of the second centrifugation was used for glycyan synthase and ADP-glucose pyrophosphorylase assays.

**PURIFICATION OF ADP-GLUCOSE PYROPHOSPHORYLASE**

**Step 1.** Frozen Synechococcus 6301 (29.3 g) were thawed into a total of 42 ml of 20 mM K-phosphate, pH 7.5, containing 5 mM diithioerythritol. The cell paste was homogenized in a Bronwill homogenizer with 30 g glass beads. The homogenate was washed once with additional buffer. The homogenate was then centrifuged at 30,000g for 15 min. The pellet was washed once in additional buffer and centrifuged. The supernatants were combined.

**Step 2.** The combined supernatants were brought to 60 C within 5 min and kept at 60 to 63 C for an additional 4 min. This heat-treated extract was then centrifuged at 30,000g for 15 min. The pellet was washed once, and the supernatants combined.

**Step 3.** The heat-treated supernatants were chromatographed on a DEAE-cellulose column (2.5 x 22 cm) equilibrated with 20 mM K-phosphate buffer, pH 7.5, containing 2 mM diithioerythritol. The enzyme was eluted with a linear gradient consisting of 1 liter of 20 mM K-phosphate, pH 7.5, containing 2 mM diithioerythritol in the mixing chamber and 1 liter of 50 mM K-phosphate, pH 6, containing 2 mM diithioerythritol and 0.4 M KCl in the reservoir chamber.

**Step 4.** The active fractions from the DEAE-cellulose chromatography were pooled and were concentrated to 43 ml in an Amicon concentrator fitted with a PM-30 membrane. The concentrated fractions were dialyzed against two changes of 5 liters of 20 mM K-phosphate buffer, pH 7.5, containing 2 mM diithioerythritol and 20% glycerol, followed by two changes of 5 liters of 50 mM tris-Cl buffer, pH 7.2, containing 0.5 mM diithioerythritol and 20% glycerol.

The enzyme was stored at -86 C and was stable for at least 3 months. Unless otherwise noted, enzyme from step 4 was used for the determination of enzyme properties.

**RESULTS**

**Enzyme Activities in Crude Homogenates.** Table I shows the specific activities of ADP-glucose pyrophosphorylase and glycyan synthase from crude extracts of Synechococcus 6301 and Aphanoapsis 6308. No activity was detected for either enzyme when UDP-glucose was substituted for ADP-glucose in the Synechococcus assays. In addition, when 10.3 units of α-amylase were included in the Synechococcus glycyan synthase assays (not shown), no glucose incorporation was observed.

**Partial Purification of ADP-Glucose Pyrophosphorylase from Synechococcus 6301.** Table II summarizes the partial purification of ADP-glucose pyrophosphorylase. The final purification was 12-fold. By step 4, the enzyme preparation did not contain detectable amounts of phosphoglucomutase. The principal protein contaminants of the preparation (46% by weight) were a mixture of phycobilins. Subsequent experiments were with enzyme from step 4. The ratio of activity in the pyrophosphorylase direction to activity in the synthesis direction was about 3:1.

**Activator Specificity.** Glycerate-3-P was the most effective type of the activators surveyed (Table III). The enzyme was also activated by other glycolytic intermediates, AMP and ADP, but to a lesser extent than by glycerate-3-P. 2-Hydroxy-4-phosphonobutyrate, 3,4-dihydroxybutyl-1-phosphonate, and 2,3-dihydroxypyrol-1-phosphonate, added at 1 mM, were without effect.

**Effect of Activator.** The effect of glycerate-3-P is to increase the V max 14 to 16-fold and decrease the S 0.5 for glucose-1-P (Fig. 1) and ATP (Fig. 2). The activator decreases the S 0.5 for glucose-1-P from 0.48 mM in the absence of glycerate-3-P to 0.14 mM in the absence of 2 mM glycerate-3-P. Similarly, the S 0.5 for ATP is decreased from 1.35 mM to 0.30 mM by glycerate-3-P. Glycerate-3-P does not effect the hyperbolic nature of either substrate saturation curve.

The kinetic behavior of MgCl 2 saturation of ADP-glucose pyrophosphorylase is highly cooperative (Fig. 3). Although glycerate-3-P has little effect on the S 0.5 for MgCl 2 (1.4 mM in the absence of glycerate-3-P and 2 mM in the presence of 2 mM glycerate-3-P), it shifts the MgCl 2 optimum from 3 mM to between 6 and 7 mM. The Hill n of 5.6 in the absence of glycerate-3-P is reduced to 2.8 in the presence of 2 mM glycerate-3-P (inset, Fig. 3).

**Inhibition of ADP-Glucose Pyrophosphorylase.** Pi was the most effective inhibitor identified. NADPH also inhibited the enzyme with an I 50 of 1.4 mM. NADH (1 mM) and 1,6-hexanediol-bisphosphate (0.125 mM) inhibited the enzyme 35 and 53%, respectively.

Pi is a partially competitive inhibitor as it decreases the V max of the enzyme and increases the S 0.5 for glucose-1-P and ATP (Figs. 4 and 5). The S 0.5 for glucose-1-P increases to 0.78 mM, and the S 0.5 for ATP increases to 3.2 mM in the presence of 0.072 mM Pi.

**TABLE I**

<table>
<thead>
<tr>
<th>Enzyme Activities in Crude Homogenates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>ADP-glucose pyrophosphorylase</td>
</tr>
<tr>
<td>+ glycerate-3-P</td>
</tr>
<tr>
<td>+ 3 mM glycerate-3-P</td>
</tr>
<tr>
<td>Glycyan synthase</td>
</tr>
</tbody>
</table>

**TABLE II**

<table>
<thead>
<tr>
<th>Purification of ADP-Glucose Pyrophosphorylase from Synechococcus 6301</th>
</tr>
</thead>
<tbody>
<tr>
<td>The assay conditions were those of assay A.</td>
</tr>
</tbody>
</table>

**TABLE III**

<table>
<thead>
<tr>
<th>Activity of Inhibition of Synechococcus 6301 ADP-Glucose Pyrophosphorylase by Various Metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>The assay conditions were those of assay B.</td>
</tr>
</tbody>
</table>

---

Copyright © 1976 American Society of Plant Biologists. All rights reserved.
glycerate-3-P and between 7.5 and 8 in the presence of 2 mM glycerate-3-P. This change in pH does not change the \( V_{\text{max}} \) in the presence of 2 mM glycerate-3-P.

The maximal stimulation by glycerate-3-P occurred at high pH. At pH 8, 2 mM glycerate-3-P increased the \( V_{\text{max}} \) 25-fold, and the \( A_{0.5} \) for glycerate-3-P was 0.112 mM. At pH 7, stimulation of \( V_{\text{max}} \) was only 8-fold, although the \( A_{0.5} \) for glycerate-3-P was 0.046 mM. The activator saturation curves were hyperbolic at both pH values. In addition to the effect on glycerate-3-P saturation, the \( S_{0.5} \) for ATP in the absence of glycerate-3-P was lower (0.51 mM) at pH 7 than at pH 8 (1.35 mM), whereas for glucose-1-P, the \( S_{0.5} \) values at pH 7 and pH 8 were 0.44 mM and 0.48 mM, respectively. The \( I_{P_i} \) for Pi in the presence of varying concentrations of glycerate-3-P was higher at pH 7 than at pH 8 (Table IV). The cumulative effect is to make the enzyme more

Pi did not change the hyperbolic nature of the saturation curves for either substrate.

The effect of Pi can be antagonized by glycerate-3-P (Fig. 6 and Table IV). The Pi saturation curves become increasingly sigmoidal and the \( I_{P_i} \) for Pi increases as the concentration of glycerate-3-P is raised.

**Effect of pH.** The pH optima for the activated and unactivated reactions differed. The pH optimum is 7 in the absence of

---

**Fig. 1.** Dependence of the rate of ADP-glucose synthesis on the concentration of glucose-1-P in the presence of varying concentrations of glycerate-3-P. The conditions were those of assay B, except that the glucose-1-P concentration was varied and glycerate-3-P was present as indicated.

**Fig. 2.** Dependence of the rate of ADP-glucose synthesis on the concentration of ATP. The conditions were those of assay B, except that the ATP concentration was varied. Glycerate-3-P was present as indicated.

**Fig. 3.** Dependence of the rate of ADP-glucose synthesis on the concentration of MgCl₂. The conditions were those of assay B, except that the MgCl₂ concentration was varied. Glycerate-3-P was present as indicated.

**Effect of phosphate on the kinetics of ADP-glucose pyrophosphorylase.** The conditions were those of assay B, except that the glucose-1-P concentration was varied. Pi was present as indicated.

**Fig. 4.** Effect of phosphate on the kinetics of ADP-glucose pyrophosphorylase. The conditions were those of assay B, except that the glucose-1-P concentration was varied. Pi was present as indicated.

**Fig. 5.** Effect of phosphate on the kinetics of ADP-glucose pyrophosphorylase. The conditions were those of assay B, except that the ATP concentration was varied. Pi was present as indicated.
Rhodospirillum rubrum enzyme (5) and decreases the $S_0.5$ of the maize endosperm enzyme (2), although it has no effect on the MgCl$_2$ saturation curve of the spinach leaf enzyme (6). In general, the enzymes are more active at lower concentrations of MgCl$_2$ if glycylate-3-P is present. It has been shown (3, 9) that illumination causes an efflux of Mg$^{2+}$ from chloroplast thylakoids. The effect of glycylate-3-P may be to make the blue-green enzyme less dependent upon analogous changes in the concentration of Mg$^{2+}$ and may be a mechanism for diverting the control of ADP-glucose pyrophosphorylase from photosynthetic activity.

The lowered $S_0.5$ for ATP, $A_{0.5}$ for glycylate-3-P, and elevated $I_{0.5}$ for Pi at pH 7 as opposed to pH 8 (Table IV) may also serve to remove the enzyme from photosynthetic control. Illumination causes an increase in the internal pH of blue-green bacteria (20). It has been estimated that if the pH of the medium is 7, then the internal pH of illuminated blue-green cells is pH 8 (M. Avron, personal communication).

It is unlikely that NADPH inhibits ADP-glucose pyrophosphorylase activity in vivo, since the cellular concentrations of NADPH are between 0.23 mM in the light and 0.04 mM in the dark (10). These values are well below the $I_{0.5}$ of the enzyme for NADPH.

**LITERATURE CITED**