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Purification and Characterization of Phosphoglycolate Phosphatase from the Cyanobacterium *Coccochloris peniocystis*[1]

Eric G. Norman and Brian Colman*

Department of Biology, York University, North York, Ontario, Canada, M3J 1P3

ABSTRACT

The properties and role of the enzyme phosphoglycolate phosphatase in the cyanobacterium *Coccochloris peniocystis* have been investigated. Phosphoglycolate phosphatase was purified 92-fold and had a native molecular mass of approximately 56 kilodaltons. The enzyme demonstrated a broad pH optimum of pH 5.0 to 7.5 and showed a relatively low apparent affinity for substrate (K_m = 222 micromolar) when compared to that from higher plants. The enzyme required both an anion and divalent cation for activity. Mn^{2+} and Mg^{2+} were effective divalent cations while Cl^- was the most effective anion tested. The enzyme was specific for phosphoglycolate and did not show any activity toward a variety of organic phosphate esters. Growth of the cells on high CO_2 and transfer to air did not result in any significant change in phosphoglycolate phosphatase activity. Competitive inhibition of *C. peniocystis* triose phosphate isomerase by phosphoglycolate was demonstrated (K_i = 12.9 micromolar). These results indicate the presence of a specific noninducible phosphoglycolate phosphatase whose sole function may be to hydrolyze phosphoglycolate and prevent phosphoglycolate inhibition of triose phosphate isomerase.

Cyanobacteria have been shown to fix CO_2 by a C_3 pathway in which the initial fixation reaction is the carboxylation of RuBP[2] by CO_2 to produce PGA, a reaction mediated by Rubisco. However, Rubisco also catalyzes the oxidation of RuBP in C_3 plants to produce PGA and phosphoglycolate, a compound that is rapidly converted to glycolate in the presence of PGPase (27). Most investigations of PGPase have been carried out on enzyme isolated from C_3 plants (3, 15, 18, 25) in which the glycolate pathway is fully operative, and carbon flow through this pathway represents a significant proportion of the carbon being fixed via photosynthesis. Cyanobacteria, however, have the capacity to take up actively both CO_2 and HCO_3^- and to accumulate high intracellular concentrations of inorganic carbon which suppresses RuBP oxidation. The synthesis of glycolate, therefore, is suppressed in these organisms and the extent to which a glycolate pathway operates is questionable (10). Studies of glycolate metabolism in cyanobacteria indicate that the pathway is incomplete (23) and that alternate pathways are involved in the metabolism of glycolate (7).

Phosphoglycolate phosphatase has not been fully characterized from any cyanobacterial species. The enzyme has been detected and partially characterized from the cyanobacteria *Anabaena variabilis* and *Anacystis nidulans* (14) and was found to have some characteristics different from those of higher plant forms of the enzyme (14). In this study, we have partially purified and characterized PGPase from the cyanobacterium *Coccochloris peniocystis*, a species that does not produce significant amounts of glycolic acid regardless of experimental or growth conditions.

MATERIALS AND METHODS

Growth of Cyanobacteria

The cyanobacterium *Coccochloris peniocystis* (No. 1548) was obtained from the University of Texas Culture Collection. Batch cultures were grown axenically as described previously (23) and were maintained in suspension by bubbling with air or 2% CO_2 in air. Medium for cells grown on 2% CO_2 was buffered with 10 mM Bicine at pH 8.0.

Preparation of Cell-Free Extracts

Cells were harvested by centrifugation at 9,000*g* for 10 min at 25°C and washed in pH 6.5 buffer containing 10 mM Mes, 10% glycerol, and 4 mM $MgCl_2$. This buffer was used throughout the entire purification unless otherwise noted. The cells were then resuspended in a minimum volume of ice-cold buffer. All subsequent steps were carried out at 4°C. The concentrated cell suspension was then ground with an equal volume of glass beads (0.10–0.11 mm) using a Vortex mixer in 10 one-minute periods allowing 1 min for cooling between mixing periods to prevent localized heating. The extract was decanted off, the beads washed with buffer, and the combined extract and washings centrifuged for 30 min at 25,000*g*. The supernatant, designated the cell-free extract, was used for subsequent purification steps.

Purification of PGPase

Solid ammonium sulfate was added to the cell-free extract to 45% saturation, the mixture equilibrated with stirring for

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[2] Abbreviations: RuBP, ribulose-1,5-bisphosphate; GA-3-P, glyceraldehyde-3-phosphate; GA-3-PDH, PFK, phosphofructokinase; PG, phosphoglycolate; PGA, phosphoglyceric acid; PGPase, phosphoglycolate phosphatase; Rubisco, ribulose-1,5-bisphosphate carboxylase oxygenase; TPI, triose phosphate isomerase.

30 min, and the precipitate removed by centrifugation for 30 min at 22,000*g*. Solid ammonium sulfate was then added to the supernatant to raise it to 65% saturation, stirred for 30 min, and then centrifuged for 30 min at 22,000*g*. The supernatant was discarded, the pellet resuspended in 1.0 mL Mes buffer containing 75 mM KCl, and dialyzed against the same buffer for 4 h. A 0.5 mL aliquot of this dialyzed preparation was then applied to a 1.0 × 100 cm Sephacryl S-200 column equilibrated with the same KCl-Mes buffer. Fractions containing PGPase activity were pooled and applied directly to a 1.0 cm × 8.0 cm DEAE cellulose column (DE-52 Whatman) equilibrated with Mes buffer. No PGPase activity was detected in the unbound elution volume. The bound enzyme was eluted with a linear gradient of KCl (0–400 mM in 60 mL in Mes buffer). The enzyme eluted at approximately 100 mM and fractions containing activity were pooled and concentrated using a Millipore CX-10 ultrafiltration unit. This concentrated enzyme preparation was used immediately for all kinetic studies or stored at −20°C for further physical studies. It was found to be stable for up to 6 months with no appreciable loss of activity.

Purification of TPI

Cell-free extracts were prepared as described for PGPase using a pH 7.2 buffer containing 50 mM Bis-Tris Propane, 5 mM EDTA, and 10% glycerol. Ice-cold acetone was added to the cell-free extract to 30%, the mixture equilibrated with stirring for 60 min, and the precipitate removed by centrifugation for 15 min at 22,000*g*. Cold acetone was then added to the supernatant to raise it to 60%, stirred for 60 min, and centrifuged for 20 min at 22,000*g*. The supernatant was discarded, the pellet resuspended in 4 mL of the Bis-Tris Propane buffer, and dialyzed against the same buffer for 20 h. This dialyzed enzyme preparation was used for studies of TPI.

Estimation of the Molecular Mass of PGPase

A Sephacryl S-200 column was equilibrated with KCl-Mes buffer (75 mM KCl) and calibrated with the following protein standards of known molecular mass: β-amylase (200 kD), alcohol dehydrogenase (150 kD), BSA (66 kD), ovalbumin (43 kD), bovine erythrocyte carbonic anhydrase (29 kD), and horse heart Cyt *c* (12.4 kD). The fraction of the stationary gel volume, available for diffusion of a given solute species (K_{av}) was determined for the molecular mass standards and for PGPase using the equation:

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

where V_e is the elution volume of the protein sample, V_0 is the void volume determined by the elution of dextran blue, and V_t is the calculated volume of the column bed. K_{av} for standards plotted against the log of molecular mass was used to estimate the molecular mass of PGPase.

Enzyme and Protein Assays

PGPase was assayed by measuring the release of phosphate from the PG using a modification of the method of Lowry and Lopez (19). The assay mixture (140 μL) contained 10 mM Mes buffer (pH 6.5), 10% glycerol, 4 mM $MgCl_2$, and 10 mM PG. The reaction, initiated by the addition of the enzyme preparation, was incubated at 25°C, and stopped by the addition of 1 mL of the reagent mixture containing 0.36% ammonium molybdate and 9% ascorbic acid in 1 N sulfuric acid. The reaction mixture was then incubated for 40 min at 45°C and the absorbance measured at 820 nm. Enzyme activity was expressed as nanomoles phosphate min^{-1} mg $protein^{-1}$.

To investigate the possible activation by ions, the PGPase enzyme preparation was first dialyzed against a buffer void of added salts. For cation activation studies, the enzyme preparation was assayed in the presence of 4 mM sulfate salts of the respective divalent cation and saturating anion (8 mM NaCl). For anion activation studies the enzyme preparation was assayed in the presence of 8 mM sodium salts of the respective anion and saturating divalent cation (4 mM $MgSO_4$). Neither the sulfate anion nor sodium cation affected activity at the concentrations used as verified by the addition of 4 mM Na_2SO_4.

Triose phosphate isomerase was assayed with GA-3-P as substrate by measuring the oxidation of NADH as described by Gibbs and Turner (11). Reaction mixtures contained 0.1 mM NADH, 50 mM Hepes (pH 7.2), 1 unit glycerol phosphate dehydrogenase, and varying concentrations of GA-3-P and PG in a final volume of 1 mL. The reaction was initiated by the addition of 20 μL of enzyme preparation, and the oxidation of NADH was monitored continuously at 340 nm using a Beckman DU-65 spectrophotometer. Enzyme activity was expressed as nanomoles min^{-1} mg $protein^{-1}$.

Protein was assayed according to a modification of the Lowry procedure (21).

All experiments were repeated at least three times. Data presented represent averages of these experiments or averages of replicates within a set of representative data.

RESULTS

Characterization of PGPase

PGPase was purified approximately 92-fold (Table I) from crude extracts of *C. peniocystis* to a specific activity of 1.65 μmol min^{-1} mg $protein^{-1}$. This enzyme preparation was reasonably stable and could be stored at −20°C for up to 6 months with no appreciable loss of activity. Native PAGE of this preparation followed by silver staining indicated that the

Table I. *Summary of the Partial Purification of PGPase from C. peniocystis*

Purification Step	Specific Activity	Purification	Recovery
	nmole min^{-1} protein^{-1}	*-fold*	%
Crude extract	17.9	1.0	100
Cell-free extract	28.5	1.6	86
45–65% $(NH_4)_2SO_4$	320.7	18.0	64
Sephacryl S-200	698.7	39.1	48
DE-52 Column	1652.5	92.6	34

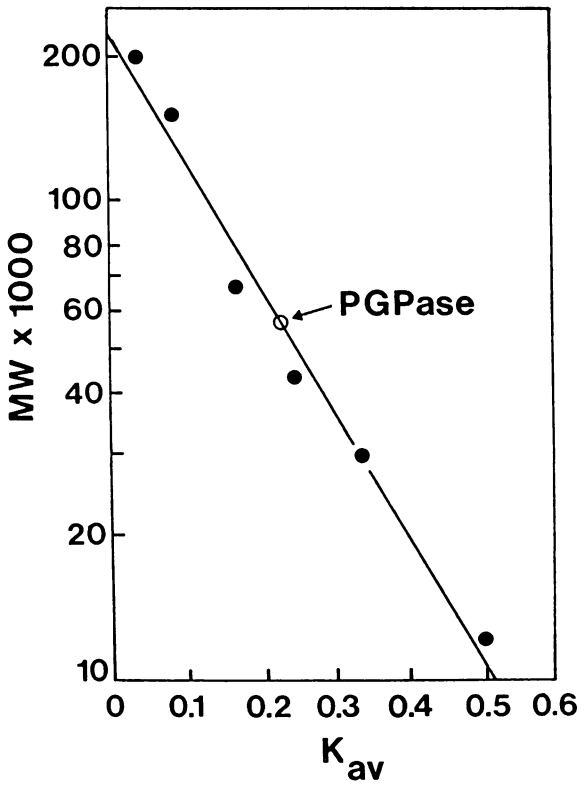

Figure 1. Estimate of molecular weight of PGPase using Sephacryl S-200 gel filtration based on the K_{av} of molecular mass standards (●) and PGPase (○). (Standard curve correlation coefficient $r = 0.982$.)

preparation was not homogenous. The PGPase from *C. peniocystis* displayed characteristics of precipitation and binding similar to those observed in the enzyme from the cyanobacterium *Anacystis nidulans* and had a comparable specific activity (14). However, the PGPase obtained from *C. peniocystis* appears to be more stable than that from *A. nidulans* (14), which may be due to the use of 10% glycerol during all stages of purification. The photosynthetic accessory pigment phycocyanin represented a significant proportion of protein through all steps of purification and significantly reduced the specific activity in the final enzyme preparation.

Using gel filtration, the molecular mass of PGPase from *C. peniocystis* was estimated to be 56 kD (Fig. 1). The enzyme eluted from a Sephacryl S-200 column as a single, symmetric peak of activity. This estimated molecular mass is very similar to that determined for tobacco PGPase (58 kD; 3) and corn PGPase (61.5 kD; 13). There is, however, a wide range of estimates for the molecular mass of PGPase from a variety of plant tissues: spinach and tobacco PGPases have been estimated to be 93 kD and 81 to 86 kD, respectively (5); *Chlamydomonas* PGPase has been estimated to be 92 kD (14); while at the lower end of the range, PGPase from wheat has been estimated to be 15 kD (26).

The purified enzyme was specific for PG and did not react with any other of the organic phosphate esters tested, including: *p*-nitrophenyl phosphate, glucose-6-phosphate, glucose-1-phosphate, fructose-1-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, 6-phosphogluconate, ribose-5-phosphate, pyridoxal-5-phosphate, 3-phosphoglyceric acid, phosphoenolpyruvate, glycerol phosphate, pyrophosphate, adenosine triphosphate, adenosine diphosphate, all of which substrates were tested at 10 mM. The crude extracts and cell-free extracts showed trace levels of alkaline phosphatase activity as determined by hydrolysis of *p*-nitrophenyl phosphate, but this activity was removed in the initial stages of PGPase purification. The high specificity of PGPase from *C. peniocystis* is consistent with PGPase from all plant tissues studied thus far and suggests a very specific role for this enzyme.

The pH profile for partially purified PGPase demonstrates a broad pH optimum from pH 5.0 to 7.5 (Fig. 2). This profile is similar to that observed for PGPase from several higher plants (6, 13, 15) and from the green alga *Chlamydomonas* (14). In contrast a very sharp pH optimum of 6.3 was observed in partially purified enzyme from both *A. nidulans* and *Anabaena variabilis* (14).

The PGPase from *C. peniocystis* showed typical Michaelis-Menten saturation kinetics for the substrate PG (Fig. 3, inset). A Lineweaver-Burk plot of these data indicates that the enzyme has a K_m of 222 μM ($r = 0.997$, Fig. 3). The K_m of PGPase from other plant tissues shows a wide range of apparent affinity from 26 μM for spinach (15) and tobacco (6) and 23 μM for *Chlamydomonas* (14) to values as high as 570 μM

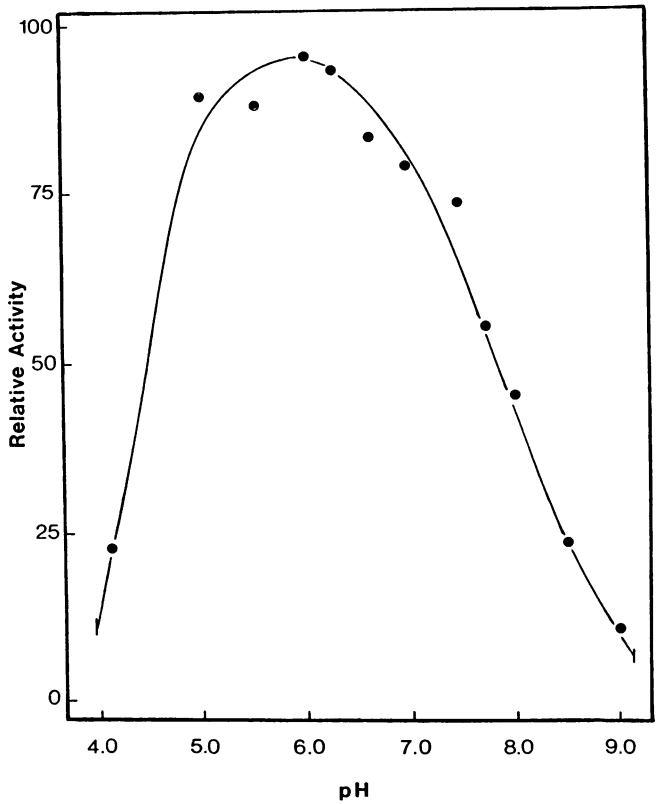

Figure 2. pH *versus* PGPase activity in partially purified enzyme preparations from *C. peniocystis*. Buffers used were at a concentration of 25 mM: phthalic acid (pH 4.1–5.0), Mes (pH 5.6–6.5), Mops (pH 6.7–7.5), Epps (pH 7.7–8.5), Bis-Tris-Propane (pH 9.0). All buffer systems contained 10% glycerol and 4 mM $MgCl_2$.

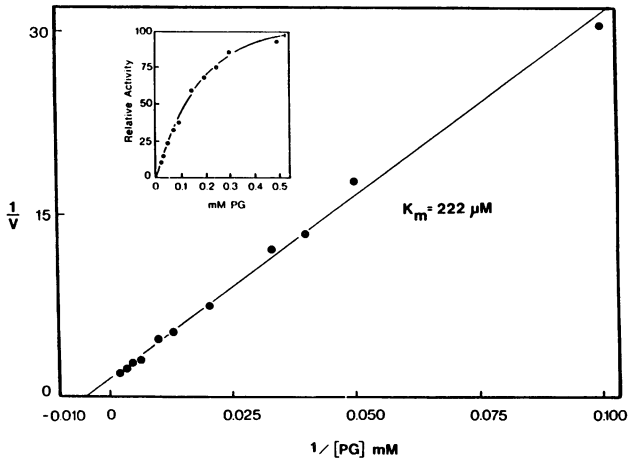

Figure 3. Lineweaver-Burk plot for PGPase estimating the K_m for PG. Inset shows PGPase activity *versus* PG concentration.

for the corn enzyme (13). The K_m values of 94 and 120 μM have been obtained for the partially purified enzyme from *A. nidulans* and *A. variabilis*, respectively (14).

Dialysis of the enzyme preparation against a buffer void of added salts rendered the enzyme essentially inactive. Activity was restored by the addition of either Mn^{2+} (100%) or Mg^{2+} (88%) to the assay mixture in the presence of 8 mM NaCl. The addition of Co^{2+} and Zn^{2+}, however, showed no detectable activation of the enzyme. The requirement for a divalent cation for catalytic activity appears to be a general characteristic of all forms of PGPase although the effectiveness of specific cations varies. A study of the *A. nidulans* enzyme (14) showed that Mg^{2+} provided maximal stimulation similar to the 88% stimulation observed in this study. Mn^{2+}, however, provided only 38% activation compared to the maximal stimulation (100%) shown here. Furthermore, Co^{2+}, which provided no activation in this study, showed a 48% stimulation of activity in *A. nidulans*. Consistent with this study was a lack of activation by Zn^{2+} in *A. nidulans*. These findings suggest that differences for a cation requirement may exist among the cyanobacteria.

Activity in the dialyzed enzyme preparation could only be restored in the presence of an anion, based on the lack of detectable activity in the absence of added anion in the presence of 4 mM $MgSO_4$. This activity was restored most effectively by the chloride anion and less so by nitrate, iodide, and glycolate (35, 32, and 30% of maximal activity, respectively). Fluoride, bicarbonate, acetate, and sulfate were all ineffective in activating the enzyme. These results are similar to the findings of Husic and Tolbert (14) where chloride and bromide provided maximal activation of PGPase in *A. nidulans* and nitrate and iodide provided activation to a lesser extent. The requirement for an anion for catalytic activity appears to be a general characteristic for PGPase from cyanobacteria, green algae (14), and higher plants (15). In this study and previous studies, activation by either the divalent cation or monovalent anion requires the presence of the other.

Effect of CO_2 Concentration During Growth

The levels of PGPase activity in cell-free extracts of *C. peniocystis* grown on high CO_2 were 27.4 nmol min^{-1} mg $protein^{-1}$ (Table II, time 0) and were not significantly different from that of air-grown cells (28.5 nmol min^{-1} mg $protein^{-1}$; Table I). PGPase activity, therefore, appears to remain constant regardless of the CO_2 concentration used during cell growth. This is consistent with studies of the cyanobacteria *A. nidulans* and *A. variabilis* and the green algae *Chlamydomonas reinhardtii* and *Chlorella vulgaris* (15), where little difference was observed between cells grown on air and on 5% CO_2 in air.

It has been shown that when *C. peniocystis* is grown on high CO_2 and transferred to air, glycolate is produced during a 30 min period as the cells adapt to air, after which glycolate production ceases (16, 24). However, when *C. peniocystis* was grown on high CO_2 and transferred to air, PGPase activity was only marginally stimulated (Table II) with no significant differences over the first 60 min. These results indicate that the levels of PGPase activity are sufficiently high, regardless of growth and incubation conditions, to metabolize all the PG produced in the cell.

Inhibition of Triosephosphate Isomerase

Triose phosphate isomerase in enzyme preparations from *C. peniocystis* had a specific activity of approximately 4400 nmol min^{-1} mg $protein^{-1}$. The enzyme demonstrated typical Michaelis-Menten saturation kinetics for the substrate GA-3-P (Fig. 4, inset). A Lineweaver-Burke plot of these data indicates a K_m of 205 μM (Fig. 3; $r = 0.959$). This apparent affinity is similar to that determined for the cytoplasmic form of the enzyme in pea (K_m = 200 μM; 1) and somewhat less than that determined for the green algae *Ankistrodesmus braunii* and *Scenedesmus acuminatus* (K_m = 434 and 970 μM, respectively; 22). In the presence of 40 μM PG there was a 4-fold increase in the K_m with no significant change in the V_{max} (Fig. 4; $r = 0.994$) which suggests competitive inhibition of the enzyme by PG. The observed change in the K_m in the presence of 40 μm PG indicates a K_i of 12.9 μM for PG, a value similar to that determined for the cytoplasmic and chloroplastic forms of the enzyme in pea (K_i = 4.1 and 15.2 μM, respectively; 1). The kinetics observed here are consistent

Table II. *Levels of PGPase Activity during Incubation on Air Levels of CO_2 following Growth on High Levels of CO_2 (2% CO_2 in air)*

Activities are expressed as the average ± the standard deviation where $n = 5$.

Incubation Time on Air Levels of CO_2	PGPase Activity*
min	*nmole min^{-1} mg $protein^{-1}$*
0	27.4 ± 2.5[a]
30	36.4 ± 4.4[ab]
60	34.9 ± 5.4[ab]
120	39.4 ± 4.0[b]

* For values with the same letter there is no significant difference at 95% confidence using analysis of variance.

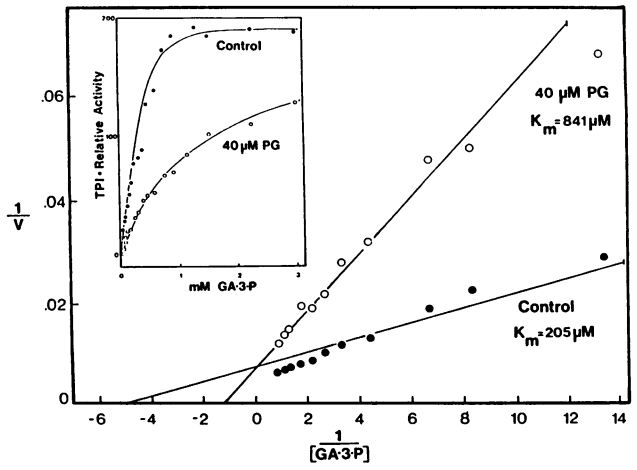

Figure 4. Lineweaver-Burk plot for TPI estimating the K_m for GA-3-P in the presence (○) and absence (●) of 40 μM PG. Inset shows reaction velocity *versus* GA-3-P concentration for TPI in the presence and absence of 40 μM PG.

with the initial studies of PG inhibition of TPI from rabbit muscle (30), where it was suggested that PG serves as an analog of the ene-diolate intermediate formed by proton transfer to the enzyme.

DISCUSSION

PGPase from the cyanobacterium *C. peniocystis* exhibits many similarities to PGPase from higher plants, green algae, and cyanobacteria. Like the enzyme from higher plants and green algae, the pH optimum is fairly broad and indicates the enzyme is operating in the cell at, or near, its optimum pH since the internal pH of *C. peniocystis* is 7.5 (8). From the K_m values determined in this study and in the studies by Husic and Tolbert (14), it is apparent that PGPase from cyanobacteria has a significantly lower apparent affinity for PG than does the enzyme from C_3 plants.

The enzyme exhibits both anion and divalent cation activation, similar to all forms of the plant enzyme studied thus far. The ion requirement for activation of PGPase in *C. peniocystis* is absolute since no activity was detected without the addition of both anion and divalent cation. Although both Mn^{2+} and Mg^{2+} activated PGPase *in vitro* in this study, Husic and Tolbert (14) have suggested that *in vivo* Mg^{2+} is likely the main cation activator, while chloride and nitrate are the main anionic activators. The kinetic properties of ion activation (14) indicate that, in general, PGPase is saturated with these activators *in vivo* and, hence, they are unlikely to play a regulatory role. It has been shown for corn PGPase that the levels of metabolites, such as NADP(H), ATP, ADP, and AMP, can regulate enzyme activity (2). The pyridine nucleotide, NADP, increased the V_{max} by 30% with no apparent change in the K_m, while increases in the adenylate energy charge stimulated activity by as much as 56%. Baldy *et al.* (2) suggest that this influence may occur via a regulatory site(s) in the protein and allow pyridine nucleotides and adenylate energy charge to tightly control PGPase activity. The extent to which this may occur in cyanobacteria is unknown.

Physiological Role of PGPase

The enzyme Rubisco, which catalyzes the oxidation of RuBP and the formation of PG, is located primarily in the carboxysomes in cyanobacteria although small amounts of soluble Rubisco are present. The K_m (CO_2) of particulate (carboxysomal) Rubisco isolated from *C. peniocystis* is significantly lower than the soluble form of the enzyme and demonstrates little or no O_2 inhibition of photosynthesis (9). Cyanobacteria are also capable of actively accumulating inorganic carbon to concentrations as high as 1000 times those in the external medium (10), conditions that would saturate Rubisco. The sequestering of Rubisco in carboxysomes combined with the active transport of inorganic carbon should significantly reduce photorespiration in cyanobacteria (10) and thus preclude the formation of PG. The presence of a highly specific PGPase, however, implies that the biosynthesis of PG by the oxidation of RuBP can occur under some conditions in *C. peniocystis* and the two other cyanobacteria studied (14). The levels of glycolate release observed in air-grown cells of *C. peniocystis* indicate there is little or no glycolate formed in these cells (24), but it is possible to stimulate RuBP oxidation by transferring high CO_2-grown cells to air levels of CO_2 when short term synthesis of glycolate occurs (16, 24). When PGPase levels were monitored following a change in CO_2 concentration, the stimulation of enzyme activity was only marginal, which indicates that the levels of PGPase are likely to be in excess of that required for the metabolism of any PG formed. The intracellular accumulation and release of glycolate from cells under conditions of changing CO_2 environments suggests that glycolate dehydrogenase may be a limiting factor in glycolate metabolism.

The presence of PGPase would be of particular significance if PG accumulation had some detrimental effect on cell metabolism. PG has been shown to inhibit the glycolytic enzyme PFK isolated from spinach chloroplasts (17) and in conjunction with inorganic phosphate may play a key role in regulating PFK activity. PG is also a known potent inhibitor of TPI from rabbit muscle (30) and pea leaf (1). PG inhibition of TPI from *C. peniocystis* has been demonstrated in this study, inhibition which would severely limit RuBP regeneration and, hence, limit photosynthesis. Following transfer from high CO_2 to air, the intracellular concentration of glycolate in *C. peniocystis* is at least 50 μM. If the activity of PGPase was low, similar concentrations of PG would accumulate and would result in the inhibition of TPI. Concentrations of PG in the 2 to 4 mM range were found in *A. variabilis* following transfer from high to low CO_2 (20) indicating that potentially inhibitory levels of PG can occur in cyanobacterial cells. In PGPase-deficient mutants of *Chlamydomonas reinhardtii* (29), barley (12), and *Arabidopsis* (28), the mutation is conditionally lethal and cultures have to be maintained on elevated levels of CO_2. The mutation in *Chlamydomonas* is particularly interesting since, like cyanobacteria, these cells also possess a CO_2-concentrating mechanism and wild-type cells display little detectable photorespiratory activity (4). It appears that the absence of PGPase represents a potentially lethal condition in photosynthetic cells. This may explain why the enzyme has been conserved even in cells that possess a

CO_2-concentrating mechanism and have the potential to suppress RuBP oxidation.

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