Structural and Regulatory Properties of Pyruvate Kinase from the Cyanobacterium *Synechococcus* PCC 6301*

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Pyruvate kinase (PK) from the cyanobacterium *Synechococcus* PCC 6301 was purified 1,300-fold to electrophoretic homogeneity and a final specific activity of 222 µmol of pyruvate produced/min/mg of protein. The enzyme was shown to have a pl of 5.7 and to exist as a 280-kDa homotetramer composed of 66-kDa subunits. This PK appears to be immunologically related to *Bacillus* PK and a green algal chloroplast PK, but not to rabbit muscle PK, or vascular plant cytosolic and plastidic PKs. The N-terminal amino acid sequence of the *Synechococcus* PK exhibited maximal (67%) identity with the corresponding region of a putative PK-A sequence deduced from the genome of the cyanobacterium, *Synechocystis* PCC 6803. *Synechococcus* PK was relatively heat-labile and displayed a broad pH optimum around pH 7.0. Its activity was not influenced by K⁺, but required high concentrations of Mg²⁺, and was relatively nonspecific with respect to the nucleoside diphosphate substrate. Potent allosteric regulation by various effectors was observed (activators: hexose monophosphates, ribose 5-phosphate, glycerol 3-phosphate, and AMP; inhibitors: fructose 1,6-bisphosphate, inorganic phosphate, ATP, and several Krebs' cycle intermediates). The enzyme exhibited marked positive cooperativity for phosphoenolpyruvate, which was eliminated or reduced by the presence of the allosteric activators. The results are discussed in terms of the phylogeny and probable central role of PK in the control of cyanobacterial glycolysis.

The capture of a photosynthetic prokaryote and its conversion into an energy-producing chloroplast was one of the key events in the evolution of the plant kingdom. Although plastid-bearing green algae and vascular plants display remarkable diversity, they can all be traced to a single successful endosymbiotic event between a cyanobacterium-like ancestor and a eukaryotic phagotroph (1). Cyanobacteria, also known as blue-green algae, are widely distributed aquatic eubacteria in which photosynthetic CO₂ fixation is mediated by the reductive pentose phosphate pathway. These organisms make a substantial contribution to global CO₂ assimilation, O₂ recycling, and N₂ fixation, and are increasingly becoming important targets for biotechnology.

In nature, most cyanobacteria face a regular cycle of light and dark. In order to meet the energy demands for maintenance and growth, they must resort to heterotrophic dark energy generation. While cyanobacterial oxygenic photosynthesis and its related metabolism have been extensively characterized, our knowledge of dark carbon metabolism and its control in cyanobacteria is comparatively sparse. In most species, glycogen accumulated during the day serves as the predominant metabolic fuel at night (2). Glucose residues derived from glycogen are catabolized via the oxidative pentose-phosphate pathway, the lower portion of glycolysis, and an incomplete Krebs' cycle, leading to the production of ATP and C-skeletons needed as anabolic precursors. However, despite the ecological, economic, and evolutionary importance of cyanobacteria, nothing is known about the properties of many potential control enzymes of their carbohydrate catabolizing pathways. One such enzyme is pyruvate kinase (PK),¹ considered to be a key regulatory enzyme of the glycolytic pathway in all the phyla.

Pyruvate kinase catalyzes the irreversible substrate level phosphorylation of ADP at the expense of PEP, producing pyruvate and ATP. It has been fully purified and extensively characterized from a wide variety of animals, plants, yeast, and non-photosynthetic bacteria where it generally exists as a homotetramer with a subunit molecular mass of 55–60 kDa. Both allosteric controls and reversible protein kinase-mediated phosphorylation may be used to coordinate the activity of animal or yeast PKs with the energy and carbohydrate demands of the cell (3–5). Similarly, bacterial (6, 7), green algal (8), and vascular plant (9, 10) PKs demonstrate tight allosteric control by a variety of metabolite effectors.

Animal and vascular plant PKs are expressed as tissue-specific isozymes that display catalytic and regulatory properties reflecting the differing metabolic requirements of the respective tissues (3–5, 10). PK isozymes, however, are not restricted to eukaryotes. For example, two types of allosteric PKs occur in *Escherichia coli* and *Salmonella typhimurium* (6, 7). In *E. coli*, PK-F is inducible and activated by Fru-1,6-P₂, whereas the PK-A is constitutive and activated by AMP and ribose-5-P (6). The latter may play an essential role to produce ATP under anaerobic conditions.

In all eukaryotic PK is cytosolic, but vascular plant and green algal PK exists as both cytosolic and plastid isozymes (PK₆ and PK₇, respectively) that differ in their respective physical, immunological, and kinetic/regulatory characteristics (8–14). Although nuclear-encoded, the plastid isozymes of most ¹ The abbreviations used are: PK, pyruvate kinase (EC 2.7.1.40); Fru, fructose; Glc, glucose; PAG, polyacrylamide gel electrophoresis; PEP, phosphoenolpyruvate; PK-A, AMP-activated pyruvate kinase; PK-F, fructose-1,6-bisphosphate-activated pyruvate kinase; PK₁, PK₁, cytosolic and plastidic pyruvate kinase isozymes, respectively; Vₘₐₓ, apparent Vₘₐₓ; MESS, 4-morpholinineethanesulfonic acid; MOPS, 4-morpholininepropanesulfonic acid; BIS-TRIS, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxyethyl)propane-1,3-diol; FPLC, fast protein liquid chromatography.
glycolytic enzymes are generally believed to have arisen from the original cyanobacterial endosymbiont, whereas the cytosolic isoforms appear to be orthologous to their animal or yeast homologs and to have been inherited from proteobacteria (15). Based upon phylogenetic analyses of the primary structure of plant, animal, and non-photosynthetic bacterial PKs, Hattori and co-workers (12) concluded that plant PKs are more similar to euobacterial homologs, than they are to eukaryotic PK. Similarly, immunological studies implied structural relatedness of a green algal PK, to *Bacillus stearothermophilus* PK, but not vascular plant or mammalian PKs (13).

Several reports have documented the PK activity of clarified cyanobacterial extracts (as cited in Ref. 2), and a genome sequencing project (16) has indicated the presence of two PK-encoding genes in the cyanobacterium *Synechocystis* PCC 6803. However, no information is available on the enzymatic properties of any cyanobacterial PK. In this study, we describe the purification to homogeneity of a cyanobacterial PK, and report the structural and regulatory properties of the purified enzyme. By analyzing PK from *Synechococcus* PCC 6301, we hope to better understand the control mechanisms governing primary carbon metabolism in cyanobacteria, and to gain insights into the structure, function, and evolutionary significance of cyanobacterial PK.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—** *Synechococcus* PCC 6301 (also known as *Synechococcus leopoliensis* and *Anacystis nidulans* R2 (17)) was obtained from the University of Toronto Culture Collection as UTCC number 102. Cells were cultured in chemostats (18) at 20 °C under a light intensity of 70 μEm−2 s−1 in modified Allen's medium (19) from which Na2CO3, Na2SiO3, 9H2O, and Fe-citrate were omitted, and to which 50 mM HEPES-NaOH (pH 8.5), 10 μM EDTA, 857 μM citric acid, and 65 μM FeSO4 were added. Chemostats were bubbled with CO2-enriched air (5%). Cells were harvested from the chemostat outflow every 3 days by centrifugation at 6,400 × g. Pellets were resuspended in 2 volumes of buffer A (see below), frozen in liquid N2, and stored at −80 °C.

**Enzyme and Protein Assays—** All solutions were prepared using Milli-Q processed water. The PK reaction was coupled to the lactate dehydrogenase reaction and assayed at 24 °C by monitoring NADH oxidation at 340 nm, in a final volume of 1 ml. Buffer A was 50 mM imidazole-HCl (pH 7.0), 25 mM MgCl2, 0.15 mM NADH, and 2 units/ml Na2SiO3 were cultured in chemostats at 20 °C under a light intensity of 70 μEm−2s−1. The lysate was clarified by centrifugation at 55,000 × g for 20 min. The clarified extract was adjusted to 15% (saturation) (NH4)2SO4, stirred for 20 min, and centrifuged as above. The supernatant was gently stirred for 30 min with 130 ml of Butyl-Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) that had been pre-equilibrated with buffer B. The slurry was poured into a 2.5 ml flowthrough column (AKTA FPLC, Amersham Pharmacia Biotech) and washed overnight at 1 ml/min with 800 ml of buffer B. Adsorbed proteins were eluted at 3 ml/min using a 520-mM linear gradient (100–0% buffer B containing 0–100% buffer C; fraction size = 20 ml). Pooled peak PK activity fractions were concentrated to 10 ml by an Amicon YM-100 ultrafilter, adjusted to 10 μg/ml chymostatin and 2 mM dithiothreitol, and dialyzed overnight against 2 liters of buffer C.

For studies of the enzymes substrate saturation kinetics and response to metabolite effectors, the stock solutions of nucleotides, PEP, organic acids, and P, were made equimolar with MgCl2, thus maintaining free Mg2+ concentrations in excess of 23 mM. Metabolite or substrate concentrations stated in the text refer to their total concentration in the assay medium unless otherwise noted.

**Purification of PK—** All procedures were carried out at 4 °C, unless otherwise noted. All buffers contained 1 mM dithiothreitol, 5 mM MgCl2, and 1 mM EDTA in addition to the following: Buffer A contained 50 mM imidazole-HCl (pH 7.2), 1 mM EGTA, 20 mM NaF, 25 mM KCl, 15% (v/v) glycerol, and 0.1% (v/v) Triton X-100. Buffer B contained 50 mM HEPES-NaOH (pH 7.1) and 15% (saturation) (NH4)2SO4. Buffer C contained 50 mM HEPES-NaOH (pH 7.1) and 10% (v/v) ethylene glycol. Buffer D contained 20 mM imidazole-HCl (pH 7.1) and 20% (v/v) glycerol. Buffer E contained 10 mM NaF, (pH 7.1) and 20% (v/v) glycerol.

Quick-frozen cells (100 g) were thawed, brought to 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, and 10 mM thioareua, and lysed by two passages through a French press at 18,000 psi (124 MPa). The lysate was clarified by centrifugation at 55,000 × g for 20 min. The clarified extract was adjusted to 15% (saturation) (NH4)2SO4, stirred for 20 min, and centrifuged as above. The supernatant was gently stirred for 30 min with 130 ml of Butyl-Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) that had been pre-equilibrated with buffer B. The slurry was poured into a 2.5 ml flowthrough column (AKTA FPLC, Amersham Pharmacia Biotech) and washed overnight at 1 ml/min with 800 ml of buffer B. Adsorbed proteins were eluted at 3 ml/min using a 520-mM linear gradient (100–0% buffer B containing 0–100% buffer C; fraction size = 20 ml). Pooled peak PK activity fractions were concentrated to 10.5 ml with an Amicon YM-100 ultrafilter, adjusted to 10 μg/ml chymostatin and 2 mM dithiothreitol, and dialyzed overnight against 2 liters of buffer C.

The sample was centrifuged as above, diluted to about 15 mg of protein/ml in buffer D, and loaded at 1 ml/min onto a column (1.1 × 9.5 cm) of Fractogel EMD DEAE-650 (S) (Merck) that had been connected to the FPLC system and pre-equilibrated with buffer D. The column was washed with buffer D until the A280 decreased to baseline. PK activity was eluted following application of a linear 0 to 500 mM KCl gradient (135 ml) in buffer D (fraction size = 5 ml). Peak activity fractions were concentrated to 1 ml as above, and desalted at 1 ml/min on a 5-ml Hi-Trap Sephadex G-50 column (Amersham Pharmacia Biotech) pre-equilibrated with buffer E.

Blue Dextran-agarose (BDA) affinity chromatography was conducted at 24 °C. The desalted sample was immediately loaded at 0.5 ml/min onto a 1 × 9 cm column of BDA (Sigma) that had been equilibrated with buffer E. The column was washed with 30 ml of buffer E, and then with 30 ml of buffer E containing 2 mM ADP (fraction size = 2.5 ml). PK activity was eluted in a broad peak (approximately 70 ml) by the application of buffer E containing 2 mM ADP and 1 mM PEP. Immediately following the collection of 4 consecutive fractions containing PK activity, they were pooled, passed through a 0.45-μm syringe filter and concentrated to 25 ml on a Superose-12 column (Superose 12/300 GL, Pharmacia Biotech) that had been equilibrated onto a Mono-Q HR 5/5 column (Amersham Pharmacia Biotech) pre-equilibrated with buffer E. The process was repeated until all PK activity eluting from the BDA column had been loaded onto the Mono-Q column.

PK activity was eluted following application of a linear 10 to 200 mM NaPi2 gradient (35 ml) in buffer E (fraction size = 0.75 ml). Peak activity fractions were pooled, concentrated to 1 ml using an Amicon PM-30 ultrafilter, divided into 20-μl aliquots, frozen in liquid N2, and stored at −80 °C until used. Purified PK was stable for at least 9 months when stored frozen. The native Mw was estimated by gel filtration FPLC on a calibrated Superose 6 HR 10/30 column as previously described (9).

**Antibody Production and Immunotitration of PK Activity—** Purification of rabbit anti-(*Synechococcus* PK) immune serum (using 180 μg of purified PK) and immunoremoval of PK activity was performed as previously described (9). Affinity-purified rabbit anti-castor (Ricinus communis) seed PK, castor seed PK, or green algal (*Selenastrum minutum* PK) IgGs and goat anti-rabbit muscle PK IgG were obtained as already reported (11, 13, 14).

**Electrophoresis and Immunoblotting—** SDS-PAGE and subunit Mw determination was performed as previously described (9). Nondenaturing PEP-PAGE was performed over the pH range 7.5 to 9.5 on precast Bio-Rad mini-gels according to the manufacturer's instructions. The enzymes Mw was determined by comparing the mobility of *Synechococcus* PK with that of six protein standards having Mw values ranging from 4.55 to 9.3.

**Immunoblotting** was performed after electrophoretic protein from SDS mini-gels to poly(vinylidene difluoride) membranes as previously
described (9, 11). Immunological specificities were confirmed by performing immunoblots in which rabbit preimmune serum was substituted for the anti-(Synechococcus PK) immune serum.

N-terminal Sequencing—Sequencing of purified PK (20 μg) was performed by automated Edman degradation at the Harvard Microchemistry Facility. Similarity searches were conducted with the BLAST program available on the National Center for Biotechnology Information World Wide Web site (24) and the GenBankTM data base.

RESULTS

Purification, and Physical, Immunological, and Structural Characterization

Purification of PK from Synechococcus PCC 6301—As shown in Table I, PK was purified 1,300-fold to a final specific activity of 222 units/mg and an overall recovery of 31%. A single peak of PK activity was obtained following all chromatographic steps. The marked hydrophobicity of the enzyme was exploited during Butyl-Sepharose FPLC of the clarified extract that resulted in a 19-fold purification (Table I). Mono-Q FPLC led to a chromatographically homogeneous preparation since the enzyme eluted from this column as a single symmetrical PK activity and A_{280} absorbing peak (results not shown).

Physical Properties—The purified enzyme was relatively heat-labile, retaining 100, 79, 58, and 0% of its activity following a 3-min incubation at 40, 50, 55, and 60 °C, respectively. Denaturation, followed by SDS-PAGE of the final preparation, resolved a single Coomassie Blue staining 66-kDa polypeptide (Fig. 1A, lanes 2 and 3), that cross-reacted strongly with the anti-(Synechococcus PK) immune serum (Fig. 1B, lane 2). Non-denaturing IEF-PAGE resolved a single Coomassie Blue staining polypeptide with a pI value of 5.7 (Fig. 1D). The native Mr was determined to be 280 ± 30 kDa (mean ± S.E., n = 3) as estimated by gel filtration FPLC on a calibrated Superose 6 column. Thus, the native PK appears to be homotetrameric.

Immunological Properties—Increasing amounts of rabbit anti-(Synechococcus PK) immune serum immunoprecipitated 100% of the activity of the purified PK. Complete immunoremoval of activity occurred at about 10 μl of immune serum per unit of PK activity. Preimmune serum had no effect on PK activity. The anti-(Synechococcus PK) immune serum could readily detect 10 pg of denatured Synechococcus PK (Fig. 1B, lane 2). Immunoblotting of 10 ng of protein from a clarified Synechococcus extract demonstrated monospecificity of the anti-(Synechococcus PK) immune serum for the 66-kDa PK subunit (Fig. 1B, lane 1). No cross-reaction was observed when immunoblots of 250 ng of the following homogeneous PK preparations were probed with this anti-(Synechococcus PK) immune serum: rabbit muscle PK, and green algal (S. minutum), or vascular plant (castor and rapeseed) PK, and PK_{c}. Similarly no cross-reaction was observed when an immunoblot of 250 ng of the purified Synechococcus PK was probed with anti-(rabbit muscle PK or rapeseed PK, or castor seed PK_{c}) IgGs. However, a cross-reaction was observed between the anti-(Synechococcus PK) immune serum and B. steatorhophilus PK (Fig. 1B, lane 3), and between the anti-green algal PK_{c} IgG and B. steatorrhophilus and Synechococcus PKs (Fig. 1C, lanes 1 and 2).

N-terminal Sequence Comparison—The N-terminal 15-residue amino acid sequence of the 66-kDa subunit of Synechococcus PK was determined and compared with its counterpart in other PKs (Fig. 2). The sequence best aligned with the corresponding region deduced from the nucleotide sequence of a putative PK-A gene from the cyanobacterium Synechocystis PCC 6803 (16). The next closest resemblance was with the N terminus of Synechocystis PK-F, followed by other bacterial PKs.

Kinetic Properties

Effect of pH—The influence of pH on the V_{max,app} of purified Synechococcus PK was determined over the pH range of 5.2 to 8.9. The enzyme exhibited a broad pH-V_{max,app} profile with a maximum occurring at approximately pH 7.0 (Fig. 3). The results indicate that the deprotonation and protonation of groups having p_{K,a} values of about 6.0 and 7.8, respectively, are needed for catalytic activity. It should be noted that these p_{K,a} values must be interpreted with caution as they may not accurately reflect the p_{K,a} of a specific ionizable group (22).

At pH 7.0 the enzyme showed equivalent activity in 50 mM imidazole, 50 mM Hepes, or a mixture of 25 mM MES and 25 mM Bis-Tris propane, whereas about 10% lower activity was obtained with 50 mM MOPS as the assay buffer. Subsequent kinetic studies were routinely conducted using 50 mM imidazole buffer at pH 7.0 and 7.5 which, respectively, correspond to the estimated intracellular pH of Synechococcus PCC 6301 in the dark and light (25).

Cofactor Requirements and Substrate Saturation Kinetics—Unlike most known PKs, the activity of Synechococcus PK was independent of a monovalent cation such as K^+, Na^+, or NH_4^+. Enzymatic activity was unaffected by KCl concentrations in the
range of 0 to 100 mM. To eliminate the presence of 0.3 mM Na\(^+\) during the PK activity determination (due to the addition of 0.15 mM Na\(_2\)SO\(_4\)-NADH to the standard coupled reaction mixture), a fixed timed assay was utilized in which NADH and lactate dehydrogenase were initially omitted. The reaction was terminated after 3 min (by boiling for 1 min) and the amount of pyruvate produced quantified spectrophotometrically at 340 nm, 10 min following the addition of 0.15 mM NADH and 2.5 units/ml of rabbit muscle lactate dehydrogenase. While Synechococcus PK activity was unaffected by the absence of added monoclonal cations, the K\(^+\)-dependent rabbit muscle PK showed no activity when parallel continuous or fixed timed PK assays were conducted in the absence of 30 mM KCl.

Tables II and III summarize the \(V_{\text{max,app}}\) and \(K_m\) or \(S_{0.5}\) values obtained for PEP, ADP, and metal cation cofactors at pH 7.0 and 7.5. As demonstrated for other PKs, the activity of the cyanobacterial enzyme showed an absolute dependence for a divalent metal cation with Mg\(^{2+}\) or Mn\(^{2+}\) fulfilling this requirement. Mg\(^{2+}\) and particularly PEP exhibited sigmoidal saturation curves, whereas Mn\(^{2+}\) and ADP followed Michaelis-Menten saturation kinetics (Tables II and III; Fig. 4). The \(V_{\text{max,app}}\) at pH 7.0 or 7.5 was about 20% lower when Mn\(^{2+}\) was substituted for Mg\(^{2+}\). At both pH values the apparent \(S_{0.5}\) value for free Mg\(^{2+}\) was slightly lower than the corresponding apparent \(K_m\) value for free Mn\(^{2+}\). Thus, catalytic efficiencies obtained with Mg\(^{2+}\) were greater than those obtained with Mn\(^{2+}\) (Table II), indicating that Mg\(^{2+}\) is the preferred divalent metal cation cofactor.

Although increasing the assay pH from 7.0 to 7.5 exerted a negligible influence on the apparent \(K_m(\text{ADP})\) (Table III), it provoked an approximate 15% reduction in the apparent \(S_{0.5}(\text{PEP})\) value without markedly altering the Hill coefficient for PEP saturation (Table II). The addition of 5% (w/v) PEG 8,000 or 20% (v/v) glycerol to the reaction mixture did not alter PEP or ADP saturation kinetics of the enzyme. This PK appears to be relatively nonspecific with respect to the nucleoside diphosphate substrate (Table III). Although \(V_{\text{max,app}}\) values obtained with saturating UDP, CDP, and GDP were either equivalent or similar to that obtained with ADP, the apparent \(K_m\) values for the alternative nucleoside substrates were up to 15-fold greater than the apparent \(K_m\) (ADP) value. Consequently, the catalytic efficiency achieved with ADP was at least 7-fold greater than the value obtained with any other nucleoside substrate (Table III), indicating that ADP is the preferred substrate for the enzyme. At concentrations greater than 5 mM, ADP became slightly inhibitory (10 mM ADP yielded about 85% of the activity achieved at 1 mM ADP). This inhibition is likely due to the interaction of PK with MgADP, as at 10 mM total ADP the concentrations of free ADP, HADP, MgHADP, and MgADP in the PK reaction mixture were estimated (22) to be 0.25, 0.09, 0.11, and 9.55 mM, respectively.

**Metabolite Effects**—A wide variety of compounds were tested as possible PK effectors at pH 7.0 and 7.5 with subsaturating concentrations of PEP and ADP (0.6 and 0.15 mM, respectively). The following compounds had little or no influence (± 15% of control velocity) on PK activity at either pH value: sucrose, mannose, Glc, Fru, dihydroxyacetone phosphate, shikimate, ADP-glucose, alanine, lysine, glycine, glutamine, glutamate, asparagine, aspartate, glycolate 2-phosphate, 2-phosphate glycerate, 3-phosphate glycerate, isocitrate, succinate, and NH\(_4\)Cl (all 5 mM); phenylalanine, tyrosine, tryptophan, and acetyl-CoA (0.5 mM each); and rutin, quercetin, and Fru-2,6-P\(_2\) (0.1 mM each). Table IV lists those compounds that significantly influenced the activity of the purified enzyme. This PK was generally more responsive to the various effectors at pH 7.0 than at pH 7.5 (Table IV).

**Activators**—Significant activators were the hexose phosphates, ribose 5-phosphate, glycerol 3-phosphate, and relatively low concentrations of AMP (Table IV). Synergistic or additive effects of activators at pH 7.0 were not observed, suggesting that they may all interact at a common site. The extent of activation was inversely proportional to PEP concentration (Tables IV and V; Fig. 4). This arises from the fact that although Glc-6-P, ribose 5-phosphate, or glycerol-3-P only slightly increased \(V_{\text{max,app}}\), they decreased the \(S_{0.5}\) (PEP) value by 4–7-fold, while eliminating or significantly reducing the positive cooperativity with respect to PEP (Table II; Fig. 4). Ribose 5-phosphate also functioned as an activator by relieving inhibition by Pi, ATP, citrate, and Fru-1,6-P\(_2\). The presence of 0.12 mM ribose 5-phosphate increased \(V_{\text{max,app}}\) from 50 to 350% (Table V). In addition, the fold activation by saturating ribose 5-phosphate was increased from about 5-fold to almost 11-fold in the presence of 2.5 mM P1.
Table II

Use of alternate nucleoside diphosphates by PK from Synechococcus PCC 6301

Invaraint cosubstrate concentration was 1 mM ADP. Hill coefficients are indicated in parentheses. Kinetic parameters for divalent metal cations are based upon their respective free (uncomplexed) concentration in the PK reaction mixture, and were determined using 50 mM Hepes-KOH as the assay buffer. All values are the means of at least four independent determinations and are reproducible to within ±10% (S.E.) of the mean value.

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>V_{max,app} (units/mg)</th>
<th>K_{m} (mM)</th>
<th>V_{max,app}/K_{m} (units/mg mM^{-1})</th>
</tr>
</thead>
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<td>ADP</td>
<td>232 (216)</td>
<td>0.095 (0.094)</td>
<td>2,442 (2,298)</td>
</tr>
<tr>
<td>CDP</td>
<td>235</td>
<td>0.69</td>
<td>341</td>
</tr>
<tr>
<td>GDP</td>
<td>177</td>
<td>0.65</td>
<td>272</td>
</tr>
<tr>
<td>UDP</td>
<td>257</td>
<td>1.47</td>
<td>161</td>
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<tr>
<td>IDP</td>
<td>105</td>
<td>1.31</td>
<td>80</td>
</tr>
</tbody>
</table>

**Table III**

Use of alternate nucleoside diphosphates by PK from Synechococcus PCC 6301.

Invaraint cosubstrate concentration was 2.5 mM PEP, and assays were conducted at pH 7.0. Kinetic constants obtained with ADP at pH 7.5 are indicated in parentheses. Hill coefficients were equivalent to 1.0 in all cases. All values are the means of at least four independent determinations and are reproducible to within ±10% (S.E.) of the mean value.

**Table IV**

Influence of various metabolites on the activity of PK from Synechococcus PCC 6301

Assays were conducted at pH 7.0 or 7.5 using subsaturating concentrations of PEP and ADP (0.6 and 0.15 mM, respectively). Enzymatic activity in the presence of effectors is expressed relative to the respective control set at 100%. All values represent means of at least four independent determinations and are reproducible to within ±10% (S.E.) of the mean value.

**Fig. 4.** Influence of several effectors on the PEP saturation kinetics of Synechococcus PCC 6301 PK. Assays were conducted at pH 7.0 in the presence of 1 mM ADP with and without effectors as shown.

(Table V). At 0.3 mM PEP, the K_{a} values for Glc-6-P and ribose 5-phosphate were extremely low, in the range of 1 to 3 μM, whereas the K_{a} (glycerol 3-phosphate) value was over an order of magnitude larger (Table V). The addition of 0.25 mM ribose 5-phosphate did not influence the apparent K_{m} for ADP.

**Inhibitors**—The most effective inhibitors were Fru-1,6-P_{2}, malate, 2-oxoglutarate, citrate, ATP, and P_{i} (Table IV). Inhibition of PK activity by these compounds was not an artifact due to Mg^{2+} chelation, since the concentration of free Mg^{2+} ions was always maintained at saturating levels (e.g., >25 mM; see “Experimental Procedures”). At concentrations in excess of 3.5 mM, AMP also functioned as an inhibitor (Table IV). Additive inhibition was observed when the following inhibitors were tested in pairs: malate, 2-oxoglutarate, citrate, Fru-1,6-P_{2}, and P_{i} (5 mM each; results not shown). This suggests that they may all interact at distinct sites on the enzyme. However, additive inhibition was not observed with 1 mM ATP, 5 mM Pi, or 5 mM AMP indicating that they may compete for a common site. At pH 7.0, Fru-1,6-P_{2}, citrate, ATP, and P_{i} functioned as inhibitors by increasing the S_{0.5} for PEP (Table II; Fig. 4), and K_{a} for Glc-6-P and ribose 5-phosphate (Table V).

**DISCUSSION**

This study was undertaken with the goals of purifying a cyanobacterial PK for the first time, and comparing it structurally and kinetically with other PKs. In non-photosynthetic eubacteria such as E. coli and S. typhimurium two PK isozymes may coexist under a wide range of nutritional states (6, 7). In contrast, only one type of allosteric PK has been found in other microorganisms such as B. stearothermophilus and Pseudomonas citronellis (26, 27). That cyanobacteria may contain PK isozymes was recently deduced via genomic sequencing of Synechocystis PCC 6803 in which two putative PK-encoding genes have been identified (16). However, during the isolation of PK from Synechococcus PCC 6301 only a single peak of activity
was resolved during all chromatographic steps, suggesting that a single PK isoform is expressed in this cyanobacteria under the culture conditions that were employed. The *Synechococcus* PK was purified to a specific activity (>200 units/mg; Table I) comparable to that of homogeneous PKs from other sources (7, 9, 11, 13, 14, 26, 27). Homogeneity of the final preparation was confirmed by SDS- and IEF-PAGE which each generated single protein-staining polypeptides (Fig. 1, A and D). Similar to PK from most other prokaryotic and eukaryotic sources, the purified enzyme was shown to have a pi value of about 5.7 and to exist as a 280-kDa homotetramer composed of 66-kDa subunits.

The structural relationship between *Synechococcus* PCC 6301 PK and other prokaryotic and eukaryotic PKs was investigated. Immunological comparison of PKs from various origins showed no cross-reaction between the anti-*Synechocystis* PK immune serum and rabbit PK or vascular plant PKc and PKp, nor between the *Synechococcus* PK and anti-(rabbit muscle PK or vascular plant PK, and PKp)-IgGs. However, a cross-reaction was observed between: (i) anti-(*Synechocystis* PK) immune serum and rabbit PK or *B. stearothermophilus* PK, (ii) anti-(green algal PKp) IgG and *Synechococcus* or *B. stearothermophilus* PKs (Fig. 1C). The immunological analysis suggests that there is conservation of a few epitopes between *Synechococcus* PK and green algal PKp, and *B. stearothermophilus* PK. However, cyanogen bromide fragmentation patterns of purified *Synechococcus* PK, *B. stearothermophilus* PK, green algal PKp, rabbit muscle PK, and vascular plant PK were distinct.2 CNBr peptide maps depend on the position and number of methionine residues in the protein. Therefore, the location of methionine residues in the *Synechococcus* PK is quite different from that of the other prokaryotic and eukaryotic PKs that were examined.

The N-terminal sequence of the *Synechococcus* 66-kDa PK subunit showed the best alignment (67% identity) with the corresponding region deduced from the nucleotide sequence of the PK-A gene from the cyanobacterium *Synechocystis* PCC 6803 (Fig. 2). The next closest resemblance was with the N termini deduced for *Synechocystis* PK-F and *E. coli* PK-A. Although the N terminus of *Synechococcus* PK appears to be conserved to varying degrees in all PKs examined to date (Fig. 2), the *Synechococcus* enzyme lacked the N-terminal domain found in mammalian PK and vascular plant PKc and PKp, as do the *Synechocystis, E. coli*, and *B. stearothermophilus* enzymes. This may be a characteristic of bacterial PKs. A comparison of the positional identities of *Synechocystis* PK-A with PK-F (Fig. 2) shows that they have about the same similarity relative to each other (45%) as they do to *B. stearothermophilus* PK. The sequence similarity rises to 62 to 65% if conservative amino acid substitutions are included. Surprisingly, plant PK does not cluster together with either PK-A or PK-F from *Synechocystis*. It has been suggested that high evolutionary rates of plant plastid-localized enzymes mask their true phylogenetic relationship (28). Overall the results imply that the *Synechococcus* PK examined here is related to PKs from cluster A, but is only distantly related to animal PKs, green algal or vascular plant PKc, and vascular plant PKp.

The purified *Synechococcus* PK was inactivated by heating at 60 °C for 3 min, as is the heat labile PKp, but not PK, from vascular plants and green algae (9, 11, 14, 29, 30). Similarly, PK-A but not PK-F, from the bacterium *S. typhimurium* is heat labile (7).

In common with many PKs, the *Synechococcus* enzyme exhibited a broad pH optimum of about pH 7.0. Thus, this PK may become more active in the dark, as cessation of photosynthetic electron transport with the light to dark transition causes the intracellular pH of *Synechococcus* PCC 6301 to decrease from about pH 7.5 to 7.0 (25). Although sensitivity of the enzyme to metabolite effectors was slightly dampened at the higher pH value (Table IV), efficiency of substrate utilization was comparable at both pH 7.0 and 7.5 (Tables II and III). As with all known PKs, the *Synechococcus* enzyme required a divalent metal cation cofactor, with Mg2+ or Mn2+ satisfying this requirement. However, a rather unusual feature was the enzymes apparent lack of dependence on a monovalent cation such as K+. Although rare, this has been reported for PK from several eubacterial and archaeal sources (27, 28, 31), as well as for at least one eukaryotic PK (from the amitochondrial protist *Trichomonas vaginalis*) (32). In contrast, the vast majority of eukaryotic PKs, including green algal and vascular plant PKc and PKp, (8, 9, 29, 30), require both a monovalent and divalent metal cation cofactor. Although ADP was the preferred cosubstrate, the cyanobacterial PK showed a broad specificity for nucleoside diphosphates (Table III), resembling other bacterial PKs.

In the darkened, aerobic state, cyanobacteria have been shown to catabolize glycosgen-derived hexose monophosphates primarily through the oxidative pentose-phosphate pathway and an incomplete Krebs’ cycle (2). It is interesting that the *Synechococcus* PK was potently activated by key intermediates of glycosgen breakdown and the oxidative pentose-phosphate pathway, Glc-6-P and ribose 5-phosphate, respectively (Tables II, IV, and V; Fig. 4). In each case, the net effect of activation would be to accelerate the conversion of glyceraldehyde-3-P to pyruvate with concomitant ATP production. The activators function by greatly increasing the activity of the enzyme at low, physiologically relevant, PEP concentrations (Table II, Fig. 4), and reducing its sensitivity to the various inhibitors (Table V). In the absence of activators, PEP shows strong positive cooperativity with the enzyme and a relatively high S0.5 and thus, PEP functions as a homotropic activator of the enzyme. In the presence of appropriate activators the positive cooperativity is abolished or greatly reduced, and the S0.5 may be lowered by almost 10-fold (Table II, Fig. 4). The activation by ribose 5-phosphate specifically affects the S0.5 for PEP, as no influence of this activator on ADP saturation kinetics was observed.

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2 J. Waller and W. C. Plaxton, unpublished data.
Likewise, *B. stearothermophilus* and *E. coli* PK-A are activated by AMP, ribose 5-phosphate, or hexose phosphates, but not by Fru-1,6-P₂ (6, 26). Activation of *Synechococcus* PK by AMP and ribose 5-phosphate (Tables II, IV, and V) is consistent with its classification as a PK-A. Similar to ribose 5-phosphate-activated PK from *E. coli* and other bacterial sources, the enzyme from *Synechococcus* PCC 6301 was potently inhibited by P_1 (Tables IV and V). However, an increase in the intracellular pool of Glc-6-P and ribose 5-phosphate may overcome monovalent cation dependence that was observed for the enzyme isolated from *E. coli* and other bacterial sources, the enzyme from *Synechococcus* PCC 6301 was potently inhibited by P_1 (Table V). P_1 is believed to contribute to the control of glycolytic flux in *Streptococcus mutans* (33). Further research is required to clarify the role of P_1 in the control of cyanobacterial glycolysis. Inhibition by Krebs’ cycle intermediates such as malate, 2-oxoglutarate, and citrate has been documented for a variety of PKs (8–10, 29) and provides a mechanism for respiratory control of this enzyme. ATP inhibition of PK is anticipated, as this compound is a product. Although low AMP concentrations stimulated *Synechococcus* PK activity, at higher, non-physiological concentrations, AMP functioned as an inhibitor (Table IV), likely by binding to the ATP inhibition site. The energy charge of *Synechococcus* PCC 6301 shows a marked transient reduction immediately following the light-dark transition (34), or when darkened aerobic cells are subjected to anoxia stress (35). Both perturbations should serve to enhance *Synechococcus* PK activity in vivo.

In conclusion, our results demonstrate that the activity of PK from *Synechococcus* PCC 6301 is modulated mainly by energy charge, feedforward activation by intermediates of glucon polymer degradation (hexose monophosphates), and oxidative pentose-phosphate pathway (ribose 5-phosphate), and feedback inhibition by several Krebs’ cycle intermediates. These observations strongly suggest that PK plays a significant role in the control of carbohydrate catabolism in cyanobacteria. Immunological evidence revealed that PK from *Synechococcus* PCC 6301 may be phylogenetically related to *Bacillus* PK and a green algal PK 

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