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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 pI 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 Mg^{2+}, 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 CO_2 fixation is mediated by the reductive pentose phosphate pathway. These organisms make a substantial contribution to global CO_2 assimilation, O_2 recycling, and N_2-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-P_2, 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 eukaryotes PK is cytosolic, but vascular plant and green algal PK exists as both cytosolic and plastid isozymes (PK_c and PK_p, respectively) that differ in their respective physical, immunological, and kinetic/regulatory characteristics (8–14). Although nuclear-encoded, the plastid isozymes of most

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[1] The abbreviations used are: PK, pyruvate kinase (EC 2.7.1.40); Fru, fructose; Glc, glucose; PAGE, polyacylamide gel electrophoresis; PEP, phosphoenolpyruvate; PK-A, AMP-activated pyruvate kinase; PK-F, fructose-1,6-bisphosphate-activated pyruvate kinase; PK_c and PK_p, cytosolic and plastidic pyruvate kinase isozymes, respectively; $V_{max,app}$, apparent V_{max}; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; BIS-TRIS, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)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 isozymes 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 PK_ps are more similar to eubacterial homologs, than they are to eukaryotic PK. Similarly, immunological studies implied structural relatedness of a green algal PK_p 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 $\mu E \cdot m^{-2} \cdot s^{-1}$ in modified Allen's medium (19) from which Na_2CO_3, $Na_2SiO_3 \cdot 9H_2O$, 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 $FeSO_4$ were added. Chemostats were bubbled with CO_2-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 N_2 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. Unless otherwise indicated, assay conditions for PK were 50 mM imidazole-HCl (pH 7.0), 2.5 mM PEP, 1 mM ADP, 30 mM $MgCl_2$, 0.15 mM NADH, and 2 units/ml desalted rabbit muscle lactate dehydrogenase. The mono(cyclohexylammonium) salts of PEP and ADP were employed. Assays were linear with respect to time and concentration of enzyme assayed. One unit of PK activity is defined as the amount of enzyme resulting in the utilization of 1 μmol of PEP/min. Protein concentrations were determined using a Coomassie Blue G-250 dye-binding method (20) with bovine γ-globulin as the protein standard.

Kinetic Studies—Kinetic studies were performed using a Dynatech MR-5000 Microplate reader and a final volume of 0.2 ml for the PK reaction mixture. Apparent V_{max} ($V_{max,app}$), K_m, or $S_{0.5}$, and Hill coefficient values for substrates and cofactors were calculated from the Hill equation fitted to a nonlinear least-squares regression computer kinetics program (21). I_{50} and K_a values (concentrations of inhibitor and activator producing 50% inhibition and activation of PK activity, respectively) were calculated using the aforementioned computer program. Stock solutions of metabolites were adjusted to pH 7.0, and the concentration of nucleotides was verified spectrophotometrically using published extinction coefficients.

The influence of pH on $V_{max,app}$ was determined using a mixture of 25 mM MES and 25 mM BIS-TRIS propane as the assay buffer, titrated to the desired pH with either KOH or HCl. The pH of kinetic assays was determined immediately following completion of each set of assays. $V_{max,app}$ values were estimated by fitting PEP saturation kinetic data to the Hill equation as described above. Estimation of pK_a values for the enzyme-PEP complex was made from a plot of log $V_{max,app}$ *versus* pH as described by Dixon and Webb (22).

Concentrations of free Mg^{2+} and Mn^{2+} were calculated based upon their respective binding to organophosphates, nucleotides, organic acids, P_i, and Cl^- ions using a computer program that automatically corrects for temperature, pH, and ionic strength (23). PK activity was independent of free Mg^{2+} concentrations in the range of 23 to 40 mM. For studies of the enzymes substrate saturation kinetics and response to metabolite effectors, the stock solutions of nucleotides, PEP, organic acids, and P_i were made equimolar with $MgCl_2$, thus maintaining free Mg^{2+} 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 $MgCl_2$, 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) $(NH_4)_2SO_4$. 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 NaP_i (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 thiourea, and lysed by two passages through a French press at 18,000 psi (124 MPa). The lysate was clarified by centrifugation at 35,000 × *g* for 20 min. The clarified extract was adjusted to 15% (saturation) $(NH_4)_2SO_4$ by the addition of finely ground $(NH_4)_2SO_4$, 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 column (3.2 × 17 cm), connected to an ÄKTA FPLC system (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-ml linear gradient (100–0% buffer B simultaneous with 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 A_{280} 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 column (1 × 6.5 cm) of BDA (Sigma) that had been pre-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 injected using a 10-ml Superloop at 0.5 ml/min onto a Mono-Q HR 5/5 column (Amersham Pharmacia Biotech) pre-equilibrated with buffer E. This 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 NaP_i 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 N_2, and stored at −80 °C until used. Purified PK was stable for at least 9 months when stored frozen. The native M_r 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—Production 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_c, castor seed PK_p, or green algal (*Selenastrum minutum*) PK_p) IgGs and goat anti-(rabbit muscle PK) IgG were obtained as already reported (11, 13, 14).

Electrophoresis and Immunoblotting—SDS-PAGE and subunit M_r determination was performed as previously described (9). Nondenaturing IEF-PAGE was performed over the pH range 3 to 10 using precast Bio-Rad mini-gels according to the manufacturers instructions. The enzymes pI was determined by comparing the mobility of *Synechococcus* PK with that of six protein standards having pI values ranging from 4.55 to 9.3.

Immunoblotting was performed after electroblotting protein from SDS mini-gels to poly(vinylidene difluoride) membranes as previously

TABLE I
Purification of PK from 100 g of chemostat-cultured Synechococcus PCC 6301

Step	Volume	Activity	Protein	Specific activity	Purification	Yield
	ml	*units*	*mg*	*units/mg*	*-fold*	%
Clarified extract	295	1,139	6490	0.13	1	100
Butyl-Sepharose	160	673	208	2.5	19	59
DEAE-Fractogel	15	689	27	19.7	150	61
BDA & Mono-Q	1.0	355	1.6	222	1,300	31

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 GenBank™ 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. 1*A*, *lanes 2 and 3*), that cross-reacted strongly with the anti-(*Synechococcus* PK) immune serum (Fig. 1*B*, *lane 2*). Nondenaturing IEF-PAGE resolved a single Coomassie Blue staining polypeptide with a pI value of 5.7 (Fig. 1*D*). The native M_r 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. 1*B*, *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. 1*B*, *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_c and PK_p. 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_c or castor seed PK_p) IgGs. However, a cross-reaction was observed between the anti-(*Synechococcus* PK) immune serum and *B. stearothermophilus* PK (Fig. 1*B*, *lane 3*), and between the anti-(green algal PK_p) IgG and *B. stearothermophilus* and *Synechococcus* PKs (Fig. 1*C*, *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.

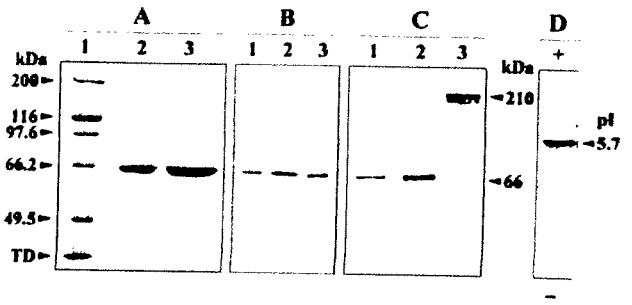

FIG. 1. **SDS-PAGE, immunoblot, and IEF-PAGE analyses of PK from *Synechococcus* PCC 6301.** *A*, SDS-PAGE (7.5% (w/v) separating gel) of purified *Synechococcus* PK. *Lane 1* contains 2 μg of various protein standards. *Lanes 2* and *3* contain 1 and 4 μg, respectively, of the final preparation. Protein staining was performed with Coomassie Blue R-250. *B* and *C*, immunoblot analyses were performed using anti-(*Synechococcus* PK)-immune serum (*B*) or anti-(green algal (*Selenastrum minutum*) PK_p)-IgG (13) (*C*). Antigenic polypeptides were visualized using an alkaline phosphatase-tagged secondary antibody and a chromogenic substrate as previously described (9, 11). Phosphatase staining was for 5 to 10 min at 25 °C. *B. Lane 1* contains 10 ng of protein from a clarified extract prepared from *Synechococcus* PCC 6301 cells. *Lane 2* contains 10 pg of purified *Synechococcus* PK. *Lane 3* contains 100 ng of *B. stearothermophilus* PK. *C. Lane 1* contains 25 ng *B. stearothermophilus* PK. *Lane 2* contains 50 ng of *Synechococcus* PK. *Lane 3* contains 25 ng of a purified green algal (*S. minutum*) PK_p, previously demonstrated to exist as an unusual 210-kDa monomer in its native state (13). *D*, nondenaturing IEF-PAGE of 5 μg of purified *Synechococcus* PK.

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 pK_a values of about 6.0 and 7.8, respectively, are needed for catalytic activity. It should be noted that these pK_a values must be interpreted with caution as they may not accurately reflect the pK_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

		% Identity in comparison to:		
		N-terminus	Entire sequence	
		Synechococcus PCC 6301	*Synechocystis* PCC 6803	
		PK	PK-A	PK-F
Syn PCC 6301	M Q P N D F Q R R T K I V A T	100		
SYN PK-A	- - T S P L P - - - - - - - -	67	100	45
SYN PK-F	K F M R P L S H - - - - - - (8)	47	45	100
BS PK	M K - K - - - - C -	40	47	43
EC PK-A	M E R R L - - - - - - T -	47	39	36
EC PK-F	M K K - - - - C -	33	42	41
HS PK-M2	D S - P I T A - N - G - I C - (40)	33	39	37
TOB PK_c	S V Y L N S P - K - - - - C - (83)	40	35	36
SOY PK_c	Q P Y D G R V P K - - - - C - (13)	33	37	36

FIG. 2. **Comparison of the N-terminal amino acid sequence of *Synechococcus* PCC 6301 PK and other PKs.** Sequences of PKs except that of *Synechococcus* PK (Syn PCC 6301) were taken from the GenBank™ data base. Origins of PK are: SYN PK-A and SYN PK-F, *Synechocystis* PCC 6803 (GenBank™ accession numbers P73534 and Q55863); BS, *B. stearothermophilus* (Q02499); EC PK-A, *E. coli* PK-A (P14178); EC PK-F, *E. coli* PK-F (P21599); HS-M2, human muscle PK (P14786); TOB PK_c, tobacco plastid PK (Q40546); and SOY PK_c, soybean cytosolic PK (Q42806). *Hyphens* denote amino acid residues that are identical to that of *Synechococcus* PK. *Underlined letters* indicate positions with the conservative substitutions Glu, Asp, Asn, and Gln; Ile, Leu, Met, and Val; Phe, Tyr, and Trp; Ala, Gly, and Pro; Ser and Thr; and Arg and Lys using the standard one-letter abbreviations. *Numbers* in *parentheses* indicate the position of the right side amino acid residues from the N terminus.

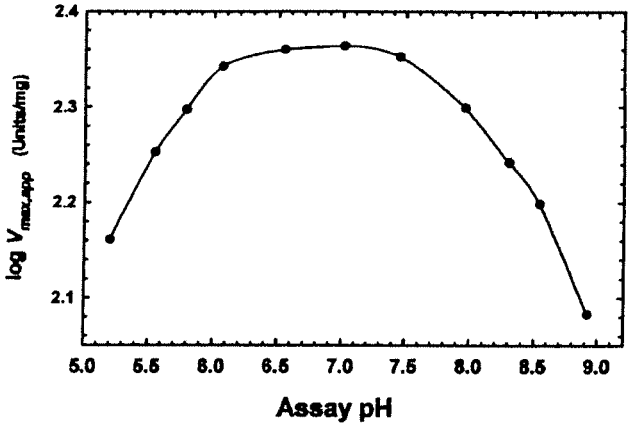

FIG. 3. **Dependence of $V_{max,app}$ on assay pH for purified PK from *Synechococcus* PCC 6301.** Enzyme assays at each pH value were buffered by a mixture of 25 mM MES and 25 mM BIS-TRIS propane, and the invariant co-substrate concentration was 2 mM. $V_{max,app}$ was estimated from fits of velocity *versus* PEP concentration to the Hill equation as described under "Experimental Procedures." Each value represents the mean of four independent determinations and is reproducible to within ± 10% (S.E.) of the mean value.

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-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 monovalent 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 $V_{max,app}$ 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 $V_{max,app}$ 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(ADP) (Table III), it provoked an approximate 15% reduction in the apparent $S_{0.5}$(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 $V_{max,app}$ 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_4Cl (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 $V_{max,app}$, 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 P_i, ATP, citrate, and Fru-1,6-P_2. The presence of 0.12 mM ribose 5-phosphate increased I_{50} values for these inhibitors 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 P_i

TABLE II

Influence of various metabolites and/or assay pH on kinetic constants of Synechococcus PCC 6301 PK for PEP and divalent cation cofactors

Invariant 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.

Variable substrate or cofactor	Addition	pH 7.0			pH 7.5		
		$V_{max,app}$	$S_{0.5}$ or K_m	$V_{max,app}/(S_{0.5}$ or $K_m)$	$V_{max,app}$	$S_{0.5}$ or K_m	$V_{max,app}/(S_{0.5}$ or $K_m)$
		units/mg	*mM*	*units/mg mM^{-1}*	*units/mg*	*mM*	*units/mg mM^{-1}*
PEP		226	0.54 (2.6)	419	210	0.45 (2.8)	467
	1 mM Ribose-5-P	246	0.13 (1.5)	1,892	ND[a]	ND	ND
	1 mM Glc-6-P	248	0.074 (1.0)	3,351	ND	ND	ND
	1 mM Glycerol-3-P	251	0.090 (1.0)	2,789	ND	ND	ND
	2.5 mM Citrate	216	0.76 (2.5)	284	ND	ND	ND
	2.5 mM Fru-1,6-P_2	220	0.78 (2.7)	282	ND	ND	ND
	2.5 mM P_i	224	0.90 (2.6)	249	ND	ND	ND
	0.5 mM ATP	212	0.75 (2.4)	283	ND	ND	ND
Mg^{2+}		236	2.9 (1.5)	81	215	1.9 (1.3)	113
Mn^{2+}		185	4.3 (1.0)	43	177	2.4 (1.0)	74

[a] ND, not determined.

TABLE III

Use of alternate nucleoside diphosphates by PK from Synechococcus PCC 6301

Invariant 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.

Nucleoside	$V_{max,app}$	K_m	$V_{max,app}/K_m$
	units/mg	*mM*	*units/mg mM^{-1}*
ADP	232 (216)	0.095 (0.094)	2,442 (2,298)
CDP	235	0.69	341
GDP	177	0.65	272
UDP	237	1.47	161
IDP	105	1.31	80

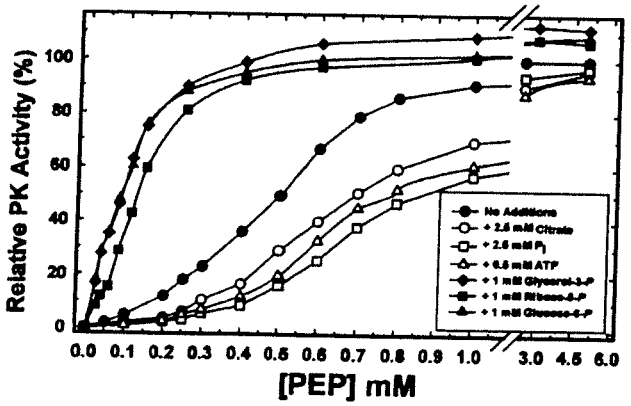

FIG. 4. **Influence of several effectors on the PEP saturation kinetics of *Synechococcus* PCC 6301 PK.** Assays were conducted at pH 7.0 in the presence of 1 mM ADP with and without effectors as shown.

(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.* >23 mM; see "Experimental Procedures"). At concentrations in excess of 3.5 mM, AMP also functioned as an inhibitor (Table IV). Additive

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.

Metabolite tested	Concentration	Relative activity	
		pH 7.0	pH 7.5
	mM		
Glc-6-P	5	139	128
Fru-1-P	5	167	140
Fru-6-P	5	142	127
Fru-1,6-P_2	5	39	66
Ribose-5-P	5	144	123
Glycerol-3-P	1	140	130
Malate	5	64	81
2-Oxoglutarate	5	76	72
Citrate	5	59	68
AMP	1	131	118
	3.5	105	96
	5	77	86
	10	15	19
ATP	1	48	59
P_i	5	58	67

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 P_i, 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

TABLE V
Kinetic constants for several effectors of PK from Synechococcus PCC 6301

Assays were conducted at pH 7.0 with subsaturating (0.3 mM) PEP. Values in parentheses indicate the maximal fold-activation of PK under the specified assay conditions. All values represent the means of at least four independent determinations and are reproducible to within ±10% (S.E.) of the mean value.

	I_{50}	K_a
	mM	μM
Glc-6-P		3.7 (5.1)
+ 2.5 mM P_i		56 (9.0)
+ 0.5 mM ATP		5.2 (6.3)
+ 2.5 mM Citrate		6.7 (6.3)
+ 2.5 mM Fru-1,6-P_2		5.4 (5.6)
Ribose-5-P		1.2 (4.5)
+ 2.5 mM P_i		20.6 (10.9)
+ 0.5 mM ATP		1.4 (5.4)
+ 2.5 mM Citrate		1.5 (5.0)
+ 2.5 mM Fru-1,6-P_2		1.7 (5.1)
Glycerol-3-P		60 (3.8)
Citrate	3.5	
+ 0.12 mM Ribose-5-P	14.4	
Fru-1,6-P_2	4.6	
+ 0.12 mM Ribose-5-P	16.0	
P_i	2.4	
+ 0.12 mM Ribose-5-P	5.9	
ATP	1.5	
+ 0.12 mM Ribose-5-P	4.4	

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-(*Synechococcus* PK) immune serum and rabbit PK or vascular plant PK_c and PK_p, nor between the *Synechococcus* PK and anti-(rabbit muscle PK or vascular plant PK_c and PK_p)-IgGs. However, a cross-reaction was observed between: (i) anti-(*Synechococcus* PK) immune serum and *B. stearothermophilus* PK (Fig. 1*B*), and (ii) anti-(green algal PK_p) IgG and *Synechococcus* or *B. stearothermophilus* PKs (Fig. 1*C*). The immunological analysis suggests that there is conservation of a few epitopes between *Synechococcus* PK and green algal PK_p, and *B. stearothermophilus* PK. However, cyanogen bromide fragmentation patterns of purified *Synechococcus* PK, *B. stearothermophilus* PK, green algal PK_p, rabbit muscle PK, and vascular plant PK_c 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 PK_c and PK_p, 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_p 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 PK_c, and vascular plant PK_p.

The purified *Synechococcus* PK was inactivated by heating at 60 °C for 3 min, as is the heat labile PK_p, but not PK_c 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 substrates 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 Mg^{2+} or Mn^{2+} 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 a least one eukaryotic PK (from the amitochondrial protist *Trichomona vaginalis*) (32). In contrast, the vast majority of eukaryotic PKs, including green algal and vascular plant PK_c and PK_p (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 glycogen-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 glycogen 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 $S_{0.5}$ 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 $S_{0.5}$ may be lowered by almost 10-fold (Table II, Fig. 4). The activation by ribose 5-phosphate specifically affects the $S_{0.5}$ for PEP, as no influence of this activator on ADP saturation kinetics was observed.

[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-P_2 (6, 26). Activation of *Synechococcus* PK by AMP and ribose 5-phosphate (Tables II, IV, and V; Fig. 4) 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_i (Tables IV and V). However, an increase in the intracellular pool of Glc-6-P and ribose 5-phosphate may overcome inhibition by P_i (Table V). P_i is believed to contribute to the control of glycolytic flux in *Streptococcus mutans* (33). Further research is required to clarify the role of P_i 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 glucan 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_p. However, the proposed cyanobacterial origin of green algal and vascular plant PK_ps (12) is difficult to reconcile with the observation that the activity of all plant or green algal PK_ps examined to date demonstrates hyperbolic PEP saturation kinetics and an absolute dependence for a monovalent cation cofactor (e.g. K^+) (8, 10, 29, 30). This contrasts with the sigmoidal PEP saturation kinetics and lack of monovalent cation dependence that was observed for the enzyme isolated from *Synechococcus* PCC 6301. Further research on cyanobacterial and plant/algal PKs may help to clarify the origin of plastid-localized PKs.

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