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Short Communication

Purification and Characterization of Phosphoribulokinase from the Cyanobacterium *Synechococcus* PCC7942

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Phosphoribulokinase (PRK) was purified to electrophoretic homogeneity from *Synechococcus* PCC7942 with high specific activity. Molecular masses of the native enzyme and its subunit were 178 and 42 kDa, respectively. Cys-17 and Cys-38 were conserved in the cyanobacterial PRK, but 18 amino acid residues between them were missing among the 40 residues found in higher plant PRKs.

Key words: Cyanobacteria — N-terminal sequence — Phosphoribulokinase (EC 2.7.1.19) — Specific activity — *Synechococcus* PCC7942.

Phosphoribulokinase (PRK; EC 2.7.1.19) catalyzes the ATP-dependent phosphorylation of D-ribulose 5-phosphate (Ru5P) to generate D-ribulose 1,5-bisphosphate (RuBP), which is a substrate for RuBP carboxylase/oxygenase (RuBisCO). PRK is a photo-regulatory enzyme and has been purified from higher plants, green algae and cyanobacteria. The specific activity (SA) of the enzyme from different sources varies considerably. The enzyme from higher plants has an SA of about 400 unit (mg protein)$^{-1}$ (Leegood et al. 1985), but enzymes from cyanobacteria have relatively low SA as compared with those of enzymes from green algae and higher plants. Moreover, the molecular masses of the subunit and native enzymes that have been reported are not in complete agreement. PRK from the cyanobacterium *Chlorogloeopsis fritschii* is a homohexamer with a molecular mass of individual subunits of 40 kDa (Marsden and Codd 1984). The PRK from *Anabaena cylindrica* has a molecular mass of 72 kDa but its subunit structure has not been determined (Serra et al. 1989). The primary structure of the enzyme from cyanobacteria has not been determined, but the amino acid sequence deduced from a cDNA sequence (Su and Bogorad 1991) revealed the two characteristic Cys residues that correspond to Cys-16 and Cys-55 in the conserved domain of PRK from spinach. Su and Bogorad pointed out that the distance between the two Cys residues in PRK from *Synechocystis* PCC6803 is 17 residues shorter than analogous distances in the eukaryotic kinases. It remains to be confirmed, however, whether the distance between the two Cys residues is a general feature of cyanobacterial PRKs. Results obtained to date also suggest that PRKs from cyanobacteria have a different structure and are more unstable than the enzymes from green algae and higher plants. This report describes the purification of PRK with high specific activity from the cyanobacterium *Synechococcus* PCC7942, the N-terminal amino acid sequence of the enzyme and the subunit structure.

Determination of PRK activity—PRK activity was measured spectrophotometrically by slightly modified version of the method of Racker (1957). The method is based on the Ru5P-dependent formation of ADP that is coupled to the oxidation of NADH in the presence of an excess of the auxiliary enzymes pyruvate kinase (PK) and lactate dehydrogenase (LDH). Decreases in absorbance at 340 nm were recorded at 25°C, in a spectrophotometer (model 430; JASCO, Tokyo). The cuvette contained, in a total volume of 1 ml, 100 μmol Tris-HCl (pH 8.0), 2 μmol ribulose-5-phosphate, 2 μmol ATP, 2.5 μmol phosphoenolpyruvate, 0.2 μmol NADH, 5 μmol GSH, 100 μmol KCl, 10 μmol $MgCl_2$, 1 unit of ribose phosphate isomerase (PRI), 4 units of PK, 1 unit of LDH and an appropriate amount of PRK.

Abbreviations: MeSH, 2-mercaptoethanol; PRK, phosphoribulokinase; Ru5P, D-ribulose-5-phosphate; RuBP, D-ribulose 1,5-bisphosphate; SA, specific activity.

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The reaction was started by the addition of PRK. One unit of activity is defined as the amount of enzyme that catalyzes the Ru5P-dependent oxidation of 1 μmol NADH in 1 min under the present conditions.

Determination of Chl content and quantitation of protein—Chl was estimated spectrophotometrically by the method of Mackinney (1941) after extraction with 80% acetone. Protein was quantitated by the method of Bradford (1976) with crystalline bovine serum γ-globulin as the primary standard.

Electrophoresis—Procedures for SDS-polyacrylamide gel electrophoresis were those described by Laemmli (1970).

Amino acid sequence analysis—The N-terminal amino acid sequence of the purified PRK was determined with an automatic protein sequencer (model PPSQ-1; Shimadzu, Kyoto). PTH-amino acids were analyzed by an on-line PTH-analyzer (model PHT 1; Shimadzu).

Antibodies—To raise antibodies against spinach PRK, the enzyme was purified by the method of Porter et al. (1986); the specific activity of the purified enzyme was 420 unit (mg protein)$^{-1}$. Antibodies against spinach PRK were raised in a rabbit by the method of Harlow and Lane (1988), and kept at 4°C with 0.02% (w/v) Thimerosal (Sigma Chemical Co., St. Louis, MO, U.S.A.). Antibodies against the purified PRK from *Synechococcus* PCC7942 were raised in a mouse by the method of Kamata et al. (1985). An eight-week-old male BALB/c mouse was immunized by injecting two doses (about 50 μg each) of the purified PRK with Freund's complete adjuvant at one-week intervals, with an additional dose of 10 μg of PRK without adjuvant 4 weeks after the first injection. One week after the final injection, the antiserum was collected and the antibodies were isolated from the antiserum with a protein A column (Amersham Laboratories, England) and used for Western blotting.

Purification of PRK—For the purification of PRK, *Synechococcus* PCC7942 was cultured photoautotrophically at 30°C in BG11 medium (Rippka et al. 1979) buffered to pH 8.0 with 10 mM HEPES-NaOH, with continuous illuminations at a photon density of 60 μmol m^{-2} s^{-1} in 8-liter batch cultures aerated at 1 liter min^{-1}. The cells were harvested by centrifugation at 4,000×*g* for 10 min at room temperature when the absorbance at 730 nm of the medium had reached 1.2 to 1.5, at the late exponential phase of cell. All steps of purification of PRK described below were carried out at 0–4°C. Cells were washed twice with 50 mM Tris-HCl (pH 8.0) containing 10 mM EDTA, 50 mM $NaHCO_3$, 10 mM MeSH, 1 mM phenylmethanesulfonyl fluoride (buffer A) and then resuspended in 30 ml of the same buffer. Cells were disrupted by a Beadbeater model 11079-00 (Biospec Products; Bartlesville, OK, U.S.A.) with 0.1 mm glass beads for 8 intervals of 30 s at 0°C. Freshly-prepared cell free extracts of *Synechococcus* PCC7942 showed PRK activity ranging from 0.19 to 0.39 unit (mg protein)$^{-1}$. The homogenate was centrifuged for 20 min at 11,000×*g*. The supernatant was treated with $(NH_4)_2SO_4$ to 30% saturation, then centrifuged for 10 min at 12,000×*g* to remove precipitate. The supernatant was made to 60% $(NH_4)_2SO_4$, and the precipitate was collected by centrifugation for 10 min at 12,000×*g*. The precipitate was dissolved in a minimum volume of 10 mM Tris-HCl (pH 8.0) containing 10 mM EDTA, 10 mM MeSH and 10% (v/v) ethylene glycol (buffer B) and dialyzed against buffer B for 12 h. The dialyzed enzyme was applied to a DEAE Sepharose CL-6B column (2×22 cm) equilibrated in buffer B. After washing with buffer B, the enzyme was eluted with a linear gradient (2×200 ml) of 100–500 mM KCl in buffer B. Fractions showing PRK activity were combined, and precipitated by 60% $(NH_4)_2SO_4$. The precipitate was collected by centrifugation for 10 min at 12,000×*g* and dissolved in 1.5 ml of 50 mM Tris-HCl (pH 8.0) containing 10 mM EDTA, 50 mM KCl and 10 mM MeSH (buffer C). Insoluble matter was spun off by centrifugation, then gel filtration was performed using a Superdex 200 column (1.6×60 cm) equilibrated in buffer C. Fractions showing PRK activity were combined, and precipitated by 70% $(NH_4)_2SO_4$. The precipitate collected by centrifugation for 10 min at 12,000×*g* was dissolved in 1 ml of 20 mM Tris-HCl (pH 8.0) containing 5 mM $MgCl_2$ and 10 mM MeSH (buffer D). The solution was passed through a Sephadex G-25 column (PD-10, Pharmacia, Uppsala, Sweden) equilibrated in buffer D to exchange buffer, and loaded onto a Blue Sepharose CL-6B column (1×9 cm). After washing with 5 column volumes of buffer D, the enzyme was eluted with a linear gradient (2×25 ml) of 0–1 M KCl in buffer D. The active fractions were collected and concentrated on a column of MonoQ HR5/5 (Pharmacia) after desalting on a column of PD-10. The buffer of the active fractions was changed to 10 mM Bicine-KOH (pH 8.0) that contained 5 mM EDTA, 10 mM DTT and 10% glycerol (v/v) with Centricon 10 (Amicon, Becerly, MA, U.S.A.).

As summarized in Table 1, the enzyme was purified about 250-fold with a recovery of 8.3% and its specific activity was 73 unit (mg protein)$^{-1}$. The enzyme was stable for at least one month at −20°C. The specific activity of the purified enzyme was the highest reported to date for the enzyme isolated from cyanobacteria (14–15 unit (mg protein)$^{-1}$, Marsden and Codd 1984, Serra et al. 1989). The main reason may be the high efficiency of affinity chromatography on Blue Sepharose. Mg^{2+} ions were required for the affinity chromatography, and the enzyme passed almost entirely through the column in the absence of this cation. The specific activity was, however, still lower than those reported for the green algal enzyme (148–450 unit (mg protein)$^{-1}$; Lazaro et al. 1986, Lin and Turpin 1992, Roesler and Ogren 1990, Roesler et al. 1992) and the enzyme from spinach (360–410 unit (mg protein)$^{-1}$; Krieger and

Table 1 Purification of phosphoribulokinase from *Synechococcus* PCC7942

Step	Total volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units mg protein^{-1})	Yield (%)
Crude extract	100	239	825	0.290	100
Ammonium sulfate fractionation (30–60%)	25	172	447	0.340	72.1
DEAE Sepharose CL-6B	21	117	49.8	2.35	49.1
Superdex 200	11	79.1	4.22	18.7	33.1
Blue Sepharose CL-6B	1	19.9	0.27	72.6	8.3

Misiorko 1986, Lavergen and Bismuth 1973, Porter et al. 1986). The differences between specific activities might also be a result of the structural diversity of PRKs, as discussed below.

Characterization of PRK—SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified enzyme yielded a single band of protein with a molecular mass of 44 kDa (Fig. 1). The molecular mass of native enzyme was determined to be 178 kDa by gel-filtration chromatography on Superdex 200. Therefore the PRK from *Synechococcus* was composed of four subunits, Marsden and Codd (1984) reported that PRK from *C. fritschii* is a hexamer and Serra et al. (1989) reported that the enzyme from *A. cylindrica* is a homo- or hetero-dimer.

Proteins that had been separated by SDS-PAGE were blotted onto an Immobilon-PVDF filter (0.45 μm; Nippon-Millipore, Tokyo) at 4 to 5 mA cm^{-2} for 40 min in 25 mM Tris-HCl (pH 8.3), that contained 200 mM glycine, in a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Lab., Richmond, CA, U.S.A.). PRKs were detected by probing the blot with antibodies, and then reaching the blot with horseradish peroxidase-conjugate and antibodies, raised

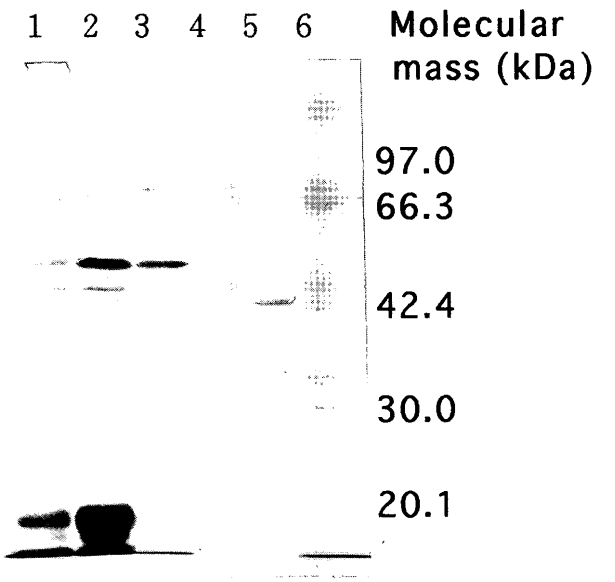

Fig. 1 SDS-PAGE of the major active fractions collected during purification of PRK from *Synechococcus* PCC7942. Lane 1, crude extract; lane 2: protein precipitated 30–60% saturation with $(NH_4)_2SO_4$; lane 3, fraction from DEAE Sepharose CL-6B; lane 4, fraction after gel filtration on Superdex 200; lane 5, fraction from Blue Sepharose CL-6B concentrated on a Mono Q column and by Centricon 10; lane 6, molecular mass markers [from top: phosphorylase *b* (97 kDa), bovine serum albumin (66.3 kDa), aldolase (42.4 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20.1 kDa)].

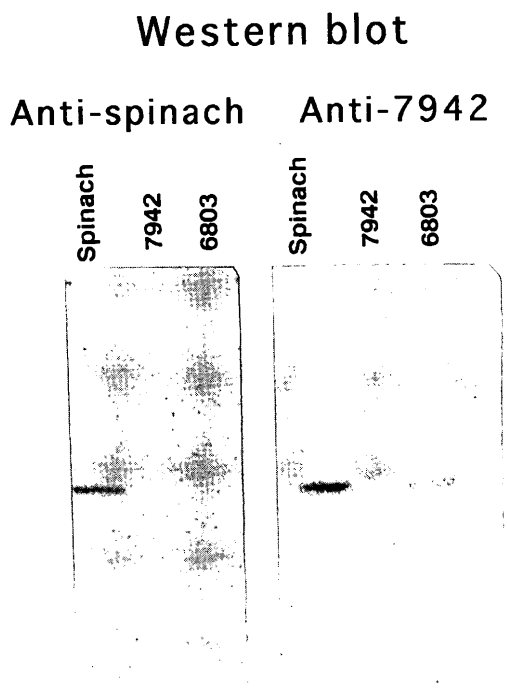

Fig. 2 Western blot analysis of spinach and cyanobacterial PRKs with antibodies against PRK (Anti-spinach) and against PRK from *Synechococcus* PCC7942 (Anti-7942). Each lane in both panels contained samples as follows: spinach, a crude extract of spinach leaves prepared according to the method of Iwaki et al. (1991); 7942, a crude extract of *Synechococcus* PCC7942; 6803, a crude extract of *Synechocystis* PCC6803. Each lane contained 2 μg of total protein.

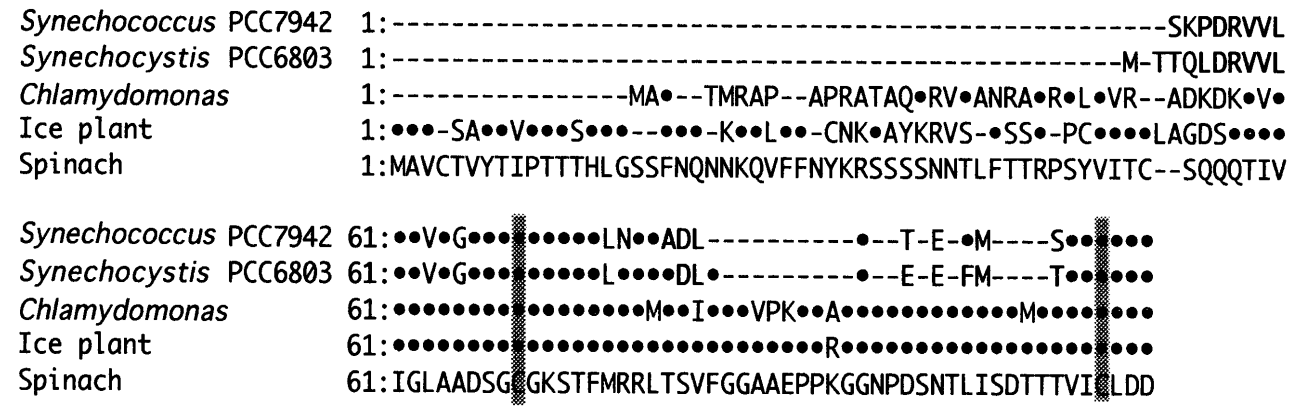

Fig. 3 Alignment of N-terminal sequences of subunits of PRK from *Synechococcus* PCC7942, *Synechocystis* PCC6803, *C. reinhardtii*, ice plant (*Mesembryanthemum crystallinum*) and spinach. Data for enzyme from *Synechocystis* PCC6803, *C. reinhardtii*, ice plant and spinach are those of Su and Bogorad (1991), Roesler and Ogren (1990), Michaelowski et al. (1992), and Roesler and Ogren (1988) and Milanez and Mural (1988), respectively. A hyphen indicates that a particular residue is not present, and a dot represents the same residue as that in the enzyme from spinach. Shaded cysteine residues are involved in regulation of the activity and in binding of ATP by the enzyme (Su and Bogorad 1991).

in goat, against mouse or rabbit IgG, by the method of Towbin et al. (1979). When antibodies against PRK from *Synechococcus* PCC7942 were used to probe an immunoblot of extracts of spinach, *Synechococcus* PCC7942 and *Synechocystis* PCC6803, it was apparent that the subunit of PRK from *Synechococcus* PCC7942 was larger than those of PRKs from spinach and *Synechocystis* PCC6803 (Fig. 2). The antibodies against PRK from spinach did not cross-react with PRKs from *Synechocystis* PCC6803 and *Synechococcus* PCC7942. Thus, there was a clear immunological difference between PRKs from spinach and the cyanobacteria, even though the molecular mass of the subunits of PRK from *Synechocystis* PCC6803 was more similar to that of the enzyme from spinach than to that of PRK from *Synechococcus* PCC7942.

The N-terminal amino acid sequence of the PRK from *Synechococcus* PCC7942 exhibited significant homology to that deduced from the cDNA sequence of the enzyme from *Synechocystis* PCC6803, but its N-terminal amino acid domain was one residue shorter than that of PRK from *Synechocystis* PCC6803 and there were 8 differences among 41 amino acid residues (Fig. 3). There are two characteristic Cys residues (Cys-17 and Cys-38) in both cyanobacterial PRKs, and they correspond to Cys-16 and Cys-55 of PRK from spinach.

Relationship between activation by light and the structure of PRK—Three groups of PRKs have been isolated from anoxygenic photosynthetic bacteria, cyanobacteria and eukaryotic photosynthetic organisms that include higher plants. PRKs from anoxygenic photosynthetic bacteria have no Cys residues in the amino-terminal region. By contrast, the two Cys residues are conserved in cyanobacterial PRKs, corresponding to the residues Cys-16 and Cys-55 in the enzyme from spinach. In the amino acid sequence between the two Cys residues, PRKs of cyanobacteria lack 17 or 18 of the 40 amino acid residues found in the enzyme from spinach, but a nucleotide-binding consensus sequence S-G-X-G-K-S-T (Higgins et al. 1986) is conserved. The absence of 17 or 18 amino acid residues might cause the only a 1.5-fold activation by light of the PRK of the cyanobacteria (Duggan and Anderson 1975), as compared to the 10-fold activation of the enzyme from spinach (Laing et al. 1981). No activation by light has been reported for PRKs from anoxygenic photosynthetic bacteria. Crawford et al. (1984) reported that the reductive pentose phosphate cycle is regulated by light in cyanobacteria but not in anoxygenic photosynthetic organisms. Buchanan (1990) noted that the conclusion of Crawford et al. is supported by the sequences of the PRK and fructose bisphosphatase (FBPase) from purple nonsulfur bacteria that were deduced from cDNAs (Gibson et al. 1990). These sequences include neither of the Cys residues required for activation by light. Tamoi et al. (1995) found that the FBPase from *Synechococcus* PCC7942 also lacked the Cys residues responsible for activation by light. The various pieces of evidence together suggest that the reductive pentose phosphate cycle in the oxygenic prokaryote *Synechococcus* PCC7942 is not strongly regulated by light, unlike the cycles in higher plants.

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