Chloroplastic phosphoglycerate kinase (PGK) was purified to homogeneity from a soluble fraction of chloroplasts of a cell-wall-deficient mutant strain of *Chlamydomonas reinhardtii* (cw-15) using ammonium sulfate fractionation, Reactive Blue-72 column chromatography, and native polyacrylamide gel electrophoresis. PGK activity was attributed to a single polypeptide with a molecular mass of 42 kDa. Relative purity and identity of the isolated enzyme was confirmed by N-terminal amino acid sequence determination. Antiserum against this enzyme was raised and a western blot analysis confirmed by N-terminal amino acid sequence determination. An anti-chloroplastic PGK serum was isolated from a *Chlamydomonas* chloroplastic PGK was isolated from a *Chlamydomonas* cDNA expression library using the anti-PGK serum. The cDNA sequence was determined and apparently codes for the entire precursor peptide, which consists of 461 codons. The results from Southern and northern blot analyses suggest that the chloroplastic PGK gene exists as a single copy in the nuclear genome of *C. reinhardtii* and is expressed as a 1.8-kb transcript. The *C. reinhardtii* chloroplastic PGK cDNA has 71 and 66% homology with wheat chloroplastic PGK and spinach chloroplastic PGK, respectively. Based on the deduced amino acid sequence, the chloroplastic PGK of *C. reinhardtii* has more similarity to plant PGKs than to other PGKs, having both prokaryotic and eukaryotic features.

PGK (EC 2.7.2.3) catalyzes the reversible conversion of 3-phosphoglycerate to 1,3-diphosphoglycerate at the expense of ATP. This enzyme is important because it functions not only in photosynthetic carbon metabolism but also in glycolysis and gluconeogenesis. In green plants this enzyme has been shown to exist in both the chloroplasts and the cytoplasm (McMorrow and Bradbeer, 1987, 1990; Secundo et al., 1990; Shah and Bradbeer, 1994). However, in most plants the major portion of PGK activity is found in the chloroplast (Shah and Bradbeer, 1994). It has been suggested from a number of biochemical studies that chloroplastic PGK may participate in a multi-component enzyme complex with other Calvin cycle enzymes, an arrangement that is postulated to be a possible mechanism to ensure the efficient CO₂ fixation by chloroplasts (Belknap and Togasaki, 1982; Gontero et al., 1988; Macioszek et al., 1990). These speculations remain tentative, however, for lack of definitive molecular and biochemical evidence.

Although a large number of cDNAs for PGK have been isolated from a variety of organisms (Watson and Littlechild, 1990), the PGK cDNAs of only two plant species have been isolated and characterized, namely those for wheat and spinach chloroplastic PGK (Longstaff et al., 1989; Bertsch et al., 1993) and a cytoplasmic PGK cDNA from wheat (Longstaff et al., 1989). Based on the deduced amino acid sequences, the three plant PGKs show significant similarity with each other and share conserved amino acid residues peculiar to prokaryotic PGKs as well as residues peculiar to eukaryotic PGKs. Although the barley chloroplastic PGK and cytoplasmic PGK have been reported to have different levels of substrate requirement (McMorrow and Bradbeer, 1987), alignment of the deduced amino acid sequences showed that amino acid residues surrounding the ligand sites of wheat chloroplastic PGK and cytoplasmic PGK were very similar to each other and to the PGKs reported from other species (Watson and Littlechild, 1990).

Recently, the first purification of a euakaryotic algal PGK was reported, with extensive biochemical characterization of two isoforms of PGK from the green alga *Selenastrum minutum* (Lin and Turpin, 1993). However, due to difficulty in isolating chloroplasts from *S. minutum*, no direct evidence for the subcellular localization of two isoforms was provided.

The further accumulation of information on the plant PGKs may be useful in helping us understand the functions of this enzyme in photosynthetic as well as in nonphotosynthetic organisms and to understand the structure and the regulatory mechanisms of chloroplastic PGK in relation to its role in the Calvin cycle. As a first step toward this goal, we chose to isolate the cDNA of chloroplastic PGK of the unicellular green alga *Chlamydomonas reinhardtii* because this photosynthetic organism has a single chloroplast per cell and is amenable to genetic and molecular analysis including transformation. Furthermore, intact chloroplasts can be isolated from this organism to facilitate the subcellular localization of enzymes.

In this paper we describe the procedure for purification of the chloroplastic PGK from *C. reinhardtii* and selected characteristics of this enzyme. We also describe the isolation and characterization of a cDNA clone coding for the chloroplastic PGK of *C. reinhardtii*.  

Abbreviations: PGK, phosphoglycerate kinase; SSC, sodium saline citrate buffer; SSPE, sodium saline phosphate EDTA buffer.
MATERIALS AND METHODS

Algal Strains and Culture Conditions

Cell-wall-deficient mutant cells of *Chlamydomonas reinhardtii* strain cw-15 (mt–) (CC-2425) were cultured in Tris-acetate-phosphate (growth) medium (Gorman and Levine, 1965) and used for protein purification and western blot analysis.

Enzyme Assay

The assay for PGK activity during protein purification was performed by linking this reaction with NADH consumption mediated by glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.2) as described by Ireland and Bradbeer (1971). Changes in *A* at 340 nm, which was proportional to the consumption of NADH and to the amount of PGK activity, were followed on a spectrophotometer (UV-160, Shimadzu). The reaction was initiated by the addition of the sample into the reaction mixture at room temperature.

Chloroplast Isolation and Soluble Protein Preparation

Intact chloroplasts were isolated from 1 × 10¹⁰ cw-15 cells as described by Belknap (1983). Cells were harvested by centrifugation (2,600g, 5 min) and disrupted by a Yeda press (equilibration at 100 p.s.i. for 3 min). The chloroplasts were isolated from disrupted cells (pressate) on a Percoll step gradient and lysed with 60 mL of ice-cold 10 mM Tris-HCl (pH 7.5) buffer. The chloroplast lysate was then centrifuged (37,600g, 30 min) to obtain the supernatant (soluble proteins) fraction.

Ammonium Sulfate Fractionation

Ammonium sulfate was added to the supernatant fraction to obtain 30% saturation. After 30 min, the precipitate was removed by centrifugation (15,000g, 30 min) and more ammonium sulfate was added to the supernatant to obtain 70% saturation. After another 30 min, the precipitate was removed by centrifugation (15,000g, 30 min) and more ammonium sulfate was added to 90% saturation. After an additional 30 min, the precipitate was collected by centrifugation (15,000g, 30 min) and suspended in 3.5 mL of 10 mM Tris-HCl (pH 7.5) buffer and desalted by dialysis against 10 mM Tris-HCl (pH 7.5) buffer.

Reactive Blue-72 Chromatography

The desalted sample from a 70 to 90% cut of ammonium sulfate fractionation was applied to a 1.5 × 14-cm column of Reactive Blue-72 agarose (total bed volume 25 mL) equilibrated with 10 mM Tris-HCl (pH 7.5) buffer (washing buffer) at a rate of about 1 mL/min. Proteins were eluted with a linear gradient of NaCl (0–200 mM) in washing buffer at the rate of 1 mL/min. The eluted fractions from the Reactive Blue-72 column that had PGK activity were pooled, concentrated, and desalted by Centriprep-10 (Amicon, Beverly, MA) as described by the manufacturer’s protocol.

Electrophoresis of Proteins

The protein sample from the Reactive Blue-72 column that had PGK activity was applied to a 7.5% native polyacrylamide gel (Laemmli, 1970) and electrophoresis was performed at 5 mA for 16 h at 4°C for further purification. Protein samples of purified protein or soluble fractions from whole-cell extracts were applied to a 7.5% SDS-polyacrylamide gel (Laemmli, 1970) and electrophoresis was performed under the same conditions as the native PAGE.

PGK Activity Staining and Protein Visualization

PGK activity staining was performed as described by Beutler (1969) with minor modifications in a 7.5% native polyacrylamide gel. After electrophoresis the gel was incubated in 30 mL of staining solution (50 mM Hepes-KOH [pH 7.8], 10 mM KCl, 1 mM EDTA [pH 7.8], 5 mM 3-phosphoglycerate, 1.4 mM NADH, 5 mM ATP, 10 mM MgCl₂, 3 units of porcine muscle glyceraldehyde phosphate dehydrogenase, final pH 7.8) and PGK activity was visualized by UV irradiation. Proteins were also visualized by staining with Coomassie blue R-250. A gel segment showing PGK activity was excised and used for N-terminal amino acid sequence analysis and antibody production.

N-Terminal Amino Acid Sequencing

After native PAGE separation, a gel segment showing PGK activity was excised and its protein contents were electroblotted to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA) as described by LeGendre and Matsudaira (1989). After electroblotting, the proteins were visualized by staining with Coomassie blue R-250 (0.1% in 50% methanol) for 5 min, followed by destaining with several changes of destaining solution (50% methanol, 10% acetic acid). The membrane containing approximately 5 μg of visualized protein was used for N-terminal amino acid sequencing. Amino acid sequence determination was carried out by the Protein Sequencing Laboratory, Genetic Engineering Facility at the University of Illinois, Urbana.

Antibody Preparation

A native polyacrylamide gel segment that contained about 50 μg of PGK was excised and used to immunize a rabbit. Booster injections were done twice using 50 μg of PGK isolated by native gel. Antibodies were prepared by Cocalico Biologicals, Inc. (Reamstown, PA).

Western Blot Analysis

For preparation of total soluble proteins, a cell suspension of cw-15 at the cell density of 6 × 10⁷ cells/mL in 10 mM Tris-HCl (pH 7.5) was sonicated (30 s, three times) at the 60% setting of Fischer Sonic Dismembrator model 150 (Pittsburgh, PA). The sonicate was centrifuged (7000g, 30 min at 4°C) and the supernatant was retained as the soluble protein fraction. Ten micrograms of soluble proteins were applied to a 7.5% SDS-polyacrylamide gel and electro-
phoresis was performed at 10 mA. Electroblotting of proteins from the gel to the nitrocellulose membrane was performed at 14 V for 14 h in transfer buffer (25 mM Tris, 190 mM Gly, 20% methanol, pH 8.3) using the Trans-Blot cell (Bio-Rad). Western blot analysis was performed as described by a commercial protocol (ECL, Amersham) using anti-PGK serum diluted 1000-fold as the primary antibodies and peroxidase-conjugated anti-rabbit Ig donkey antibodies (Amersham) as the secondary antibodies.

**cDNA Library Screening and Sequencing**

A λ gt11 C. reinhardtii cDNA expression library was obtained from Dr. S. Merchant (Merchant and Bogorad, 1987). The cDNA library was screened as described by Williams et al. (1986) using anti-PGK serum. DNA sequencing was done by the chain termination method using single-stranded templates of fragments cloned in the M13 phage vector. Both strands of the coding region were sequenced using synthesized oligonucleotide primers. Dideoxy sequencing reactions were done using a T7 DNA polymerase Sequenase kit (Sequenase version 2.0, United States Biochemical). Sequence analysis was performed using the GCG sequencing analysis software package version 7 of the Genetic Computer Group (Devereux et al., 1984).

**Southern Blot Analysis**

Genomic DNA was isolated from a 1-mL culture containing 3 × 10^6 cw-15 cells as described by Davies et al. (1992). Cells were collected by centrifugation (7000g, 5 min), suspended in 300 μL of sterile distilled water, and lysed by the addition of 300 μL of lysis buffer (100 mM Tris-HCl, pH 8.0, 40 mM EDTA, 400 mM NaCl, 2% SDS). The resulting suspension was extracted twice with phenol:chloroform: isoamyl alcohol (25:24:1) and twice with chloroform: isoamyl alcohol (24:1). Nucleic acids were precipitated by adding 2.5 volumes of 100% ethanol and cooling the sample at -80°C overnight. The recovered nucleic acids were washed with 70% ethanol and dissolved in 10 μL of sterile distilled water. Isolated DNA was digested with restriction enzymes overnight, electrophoresed in a 0.8% agarose gel in Tris-acetate-EDTA buffer, and transferred to a nylon membrane (Nytran, Schleicher & Schuell) as described by Sambrook et al. (1989) using radiolabeled Chlamydomonas PGK cDNA as a probe. Hybridization was performed in 5X SSPE, 2.5X Denhardt's solution, 1% SDS, 100 μg/mL yeast tRNA, 50% formamide at 42°C for 16 h. The filter was washed twice for 5 min each in 1X SSPE, 0.1% SDS at room temperature and for 60 min at 60°C in 0.2X SSPE, 0.1% SDS.

**RESULTS**

**Purification of Chloroplastic PGK**

Chloroplastic PGK of C. reinhardtii was purified for N-terminal amino acid sequence determination and antibody production. A summary of the purification scheme of chloroplastic PGK is shown in Table I. The initial concentration and fractionation of the soluble protein from isolated chloroplasts were achieved by precipitation with ammonium sulfate. This resulted in a 6-fold purification. The 70 to 90% ammonium sulfate fraction was passed over Reactive Blue-72 agarose, followed by elution with a NaCl gradient (Fig. 1). Fractions 25 to 27, which had PGK activity, were pooled, concentrated, and desalted. This achieved a 758-fold purification. The concentration of NaCl that eluted PGK from Reactive Blue-72 agarose was about 125 mM. Overall, 4852-fold purification of PGK was achieved. Proteins in pooled fractions were subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel. SDS-PAGE analysis showed a single major band that had a molecular mass of 42 kDa (Fig. 2A). Proteins in pooled fractions were separated by 7.5% native PAGE in preparation for N-terminal amino acid sequencing and antibody production. Figure 3 shows

| Table I. Purification of chloroplastic PGK of Chlamydomonas |
|-----------------------|-------------------|----------------|-------------------|-----------------|
| **Step**              | **Total Activity**| **Total Protein** | **Specific Activity** | **Yield** | **Purification** |
|                       | **units** | **mg** | **units/mg** | **%** | **-fold** |
| Crude stromal extract | 297      | 331   | 0.9         | 100  | 1        |
| Ammonium sulfate fractionation | 190      | 33    | 5.8         | 64   | 6.4      |
| Reactive Blue-72 chromatography | 131      | 0.03  | 4367 | 44   | 4852     |

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the result of PGK activity staining and Coomassie blue staining after native PAGE. In both cases a single band of the same mobility can be seen in pooled fractions from the Reactive Blue-72 column. Based on these data we concluded that PGK activity was attributed to this protein that had an estimated molecular mass of 42 kD. A native polyacrylamide gel segment showing PGK activity was excised and used for further experiments.

N-Terminal Amino Acid Sequencing

N-terminal amino acid sequence of the chloroplastic PGK was determined and is shown in Figure 4. The N-terminal amino acid sequence of C. reinhardtii chloroplastic PGK has 81, 77, 80, and 63% identity with the corresponding regions of wheat chloroplastic PGK, spinach chloroplastic PGK, S. minutum PGK, and S. minutum PGK2, respectively. Based on these data, we confirmed the purity and identity of our purified chloroplastic PGK of C. reinhardtii.

Western Blot Analysis

Figure 2B shows the results of a western blot analysis of soluble proteins isolated from a whole-cell extract of cw-15 using anti-PGK serum. One polypeptide with a calculated molecular mass of 42 kD was detected. This molecular mass is identical to the value obtained for the purified...
chloroplastic PGK by SDS-PAGE (Fig. 2A). Based on these data, it was concluded that this 42-kD peptide represents the chloroplastic PGK of C. reinhardtii. Since the background in the western blot analysis with the anti-PGK serum was very low, the anti-PGK serum was deemed suitable for the screening of a cDNA expression library.

**Isolation of a cDNA Clone for Chloroplastic PGK of C. reinhardtii**

Clones (5 x 10⁵) of a gt11 cDNA expression library (Merchant and Bogorad, 1987) were screened with the anti-PGK serum as described by Williams et al. (1986). One immunoreactive clone of C. reinhardtii was recovered that codes for the chloroplastic PGK of C. reinhardtii (Lin and Turpin, 1993) and C. reinhardtii chloroplastic PGK (C. reinhardtii chl).

**Structure of the Chlamydomonas Chloroplastic PGK**

The coding sequence of the cDNA clone for the precursor peptide of the chloroplastic PGK contains 461 codons and this has two coding segments. One is for the mature PGK and the other is for the transit peptide. A cleavage site for the removal of the transit peptide is between Ala at 60 and Val at 61. This cleavage site designation is based on the N-terminal amino acid sequence of purified C. reinhardtii chloroplastic PGK (Fig. 4). The mature (processed) protein contains 401 residues, whereas the transit peptide contains 60 amino acids. This predicts a calculated mol wt of 49,007 for the precursor protein and a calculated mol wt of 42,745 for the mature protein. However, in this study no peptide corresponding to the precursor protein was detected in western blot analysis. The deduced amino acid sequence for the chloroplastic PGK has 71, 69, and 66% homology with wheat chloroplastic PGK, respectively.

**Figure 4.** Alignment of the N-terminal amino acid sequence of Chlamydomonas chloroplastic PGK with those published for other organisms. Residues that are identical in more than three species are boxed. The data of the N-terminal amino acid sequences were derived from the following sources: wheat chloroplastic PGK (Wheat chl) (Longstaff et al., 1989); spinach chloroplastic PGK (Spinach chl), (Bertsch et al., 1993); and two types of PGKs (PGK1 and PGK2) of S. minutum (S. minutum PGK1 and S. minutum PGK2) (Lin and Turpin, 1993) and C. reinhardtii chloroplastic PGK (C. reinhardtii chl).

**Figure 5.** Sequence of cDNA containing the coding sequence of Chlamydomonas chloroplastic PGK. The arrow shows the starting point of the mature enzyme. The N-terminal amino acid sequence that was determined by microsequencing of the purified chloroplastic PGK is underlined.
plant PGKs and C. reinhardtii chloroplastic PGK to yeast and Thermus thermophilus PGK are relatively low.

PGKs from most species isolated so far can be divided into two groups, eukaryotic type and prokaryotic type. This classification is based on conserved amino acids that are unique among PGKs from eukaryotic sources versus those conserved among PGKs from prokaryotic sources. Figure 6 shows the alignment of the deduced amino acid sequences of several PGKs including yeast PGK as an example of a eukaryotic-type PGK, and the PGK from T. thermophilus as an example of a prokaryotic-type PGK.

Figure 6. Alignment of the deduced sequence of Chlamydomonas chloroplastic PGK with those published for other organisms. Residues that are identical in all six species are boxed. The closed circles show conserved residues at the putative ligand binding sites. The sequence data were derived from the following sources: Saccharomyces cerevisiae (Yeast) (Watson et al., 1982); T. thermophilus (Bowen et al., 1988); wheat chloroplastic PGK (Wheat chl) (Longstaff et al., 1989); wheat cytoplasmic PGK (Wheat cyt) (Longstaff et al., 1989); spinach chloroplastic PGK (Spinach chl) (Bertsch et al., 1993).

Phylogenetic analysis of the deduced amino acid sequence of C. reinhardtii chloroplastic PGK also showed the evolutionary relationships among those of several PGKs including yeast PGK, wheat PGK, and spinach PGK. For example, Chlamydomonas chloroplastic PGK lacks the “nose” region (amino acids 129-141 of yeast PGK) that can be seen in all PGK genes from eukaryotes but in none of the prokaryotes studied so far. Also, Leu at position 183 and Val at position 209 of Chlamydomonas chloroplastic PGK are conserved in the analogous regions of PGKs from prokaryotic organisms but not in PGKs of eukaryotic organisms. On the other hand, Leu’s at positions 138 and 386 of Chlamydomonas chloroplastic PGK indicate a conserved eukaryotic feature of Chlamydomonas chloroplastic PGK.

Although little amino acid sequence similarity was found between the transit peptide of Chlamydomonas chloroplastic PGK and other known transit peptides, they share some common features. For example, they are rich in the hydroxylated amino acids such as Ser and small hydrophobic amino acids such as Ala and Val (Keegstra and Olsen, 1989; Longstaff et al., 1989).

Southern Blot Analysis and Northern Blot Analysis

A single band is visible in the BgIII- and SnaBI-digested nuclear DNA (Fig. 7) on the Southern blot analysis. Since there are no sites for BgIII and SnaBI sites in cDNA of PGK, this suggests that there is a single copy of the chloroplastic PGK gene in C. reinhardtii. Although there is one BamHI site in cDNA of PGK, only a single band was evident. Since the BamHI site is located close to the 3’ end of this clone, it is possible that the other expected band of small size could...
Table II. Identities matrix of PGKs

A percentage of the number of sites occupied by identical amino acids in pairwise comparison of PGK sequences. The sequence data were derived from the following sources: spinach chloroplastic PGK (Spinach chl), (Bertsch et al., 1993); wheat chloroplastic PGK (Wheat chl) (Longstaff et al., 1989); wheat cytoplasmic PGK (Wheat cyt) (Longstaff et al., 1989); yeast PGK (Yeast) (Watson et al., 1982); T. thermophilus PGK, (Bowen et al., 1988); and C. reinhardtii chloroplastic PGK (C. reinhardtii chl).

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not be detected. Alternatively, the absence of a second BamHI fragment could be due to there being very little probe for that region. Northern blot analysis of total RNA of C. reinhardtii (data not shown) shows a single transcript (1.8 kb). This also suggests a single species of chloroplastic PGK in C. reinhardtii.

**DISCUSSION**

Chloroplastic PGK of C. reinhardtii was purified and analyzed. The estimated molecular mass of this protein by SDS-PAGE (Fig. 2) and western blot analysis (Fig. 5) was 42 kD. This was also confirmed by the deduced amino acid sequence based on the cDNA sequence of PGK. This molecular mass is in agreement with the molecular mass of other species of PGKs. More than 20 species of PGKs have been analyzed and all of these are monomers with an average molecular mass of about 40 kD (Watson and Littlechild, 1990).

The cDNA of wheat cytoplasmic PGK is the only characterized plant cytoplasmic PGK cDNA. Based on the deduced amino acid sequence, both chloroplastic PGK and cytoplasmic PGK have similar structures in wheat. In C. reinhardtii, western blot analysis (Fig. 5) could not detect any other peptides beside the chloroplastic PGK (42 kD) using a 7.5% SDS-PAGE system. One possibility is that in C. reinhardtii two types of PGK have different structures and there is no immunological cross-reactivity between chloroplastic and cytoplasmic PGK. The other possibility is that mobility of both PGKs was similar so that two peptides could not be separated by 7.5% SDS-PAGE.

Only a single copy of PGK gene was detected by Southern blot analysis using the cDNA clone of chloroplastic PGK of C. reinhardtii as a probe (Fig. 7). Northern blot analysis detected a single transcript of PGK of 1.8 kb. These data suggest that there is only one gene coding for chloroplastic PGK in C. reinhardtii. However, we cannot eliminate the possibility that several PGK genes are located in tandem.

*Chlamydomonas* chloroplastic PGK has both eukaryotic and prokaryotic features like the other three species of plant PGKs (Fig. 6). In addition, this is consistent with the wide immunological cross-reactivity of antiserum against purified *S. minutum* PGK (Lin and Turpin, 1993). Longstaff proposed the possibility that plant PGKs arose as a result of recombination between two genes, namely chloroplastic PGK and cytoplasmic PGK, based on the shared prokaryotic and eukaryotic features of both genes (Longstaff et al., 1989). The future isolation and characterization of a chloroplastic PGK gene from *Chlamydomonas* may provide additional evidence for such a possibility, as is suggested by the close similarity of the *Chlamydomonas* chloroplastic PGK gene to plant PGK genes. Our findings with the algal sequence suggest that this unique structural feature of PGK may be a conserved characteristic not only of higher plant PGKs but of photosynthetic eukaryotes in general.

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**LITERATURE CITED**


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