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Characteristics and Sequence of Phosphoglycolate Phosphatase from a Eukaryotic Green Alga *Chlamydomonas reinhardtii**

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Phosphoglycolate phosphatase (PGPase), a key enzyme of photorespiration in photosynthetic organisms, was purified from *Chlamydomonas reinhardtii*. The enzyme was an ~65-kDa homodimer with a pI value of 5.1 composed of ~32-kDa subunits not connected by any S–S bridges. It was also highly specific for phosphoglycolate with a K_m value of 140 μM and an optimal pH between 8 and 9. The activity was strongly inhibited by $CaCl_2$, and it recovered competitively following the addition of $MgCl_2$ or EGTA. A mobility shift was observed in SDS-polyacrylamide gel electrophoresis by the addition of $CaCl_2$, indicating that the enzyme binds to Ca^{2+}. The N-terminal region of amino acid sequence deduced from cDNA sequence that was not contained in the purified PGPase had similar characteristics to those of typical stroma-targeting transit peptides in *C. reinhardtii*. The following region of the deduced sequence containing 302 amino acid residues was similar to *p*-nitrophenylphosphatase-like proteins, although the purified PGPase did not hydrolyze *p*-nitrophenylphosphate. Genomic DNA fragments from wild type containing the sequence homologous to the cDNA for PGPase complemented the PGPase-deficient mutant *pgp1*. Possible regulatory mechanisms during adaptation to limiting CO_2 were discussed based on the characteristics of the purified PGPase and the deduced amino acid sequence.

Phosphoglycolate phosphatase (PGPase,[1] EC 3.1.3.18), a photorespiratory enzyme that catalyzes the hydrolysis of phosphoglycolate, which is produced by the ribulose-1,5-bisphosphate oxygenase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase, is essential for the growth of photosynthetic organisms in the light. PGPase-deficient mutants so far isolated only from *Arabidopsis thaliana* (1), *Hordeum vulgare* (2), and *Chlamydomonas reinhardtii* (3) cannot grow under ambient air (0.04% CO_2 and 21% O_2) and require elevated levels of CO_2. This is most likely because phosphoglycolate, which accumulates during photosynthesis under ambient air (4), strongly inhibits the enzyme triosephosphate isomerase (5–7).

PGPase has been partially purified from several species of plants and algae including tobacco (8, 9), spinach (8, 10), maize (11), pea (12), *Halimeda cylindracea* (13), *C. reinhardtii* (14), and *Coccochloris peniocystis* (7). PGPase has also been partially purified from components of animal tissues such as human red blood cells (15). The remarkable similarity in the kinetic characteristics of these animal PGPases to those of plant PGPases has been described previously (16). No information, however, is available on the molecular structure (*e.g.* amino acid sequences) in eukaryotes.

The regulatory mechanism of PGPase activity is still not clear in any organisms, although some observations suggest environmental regulation by such factors as CO_2 concentration (3, 17, 18) and light (19). PGPase also seems to have an important role in animals by affecting the phosphoglycolate level. In human red blood cells, for example, phosphoglycolate is an effective activator of the bisphosphoglycerate shunt, which is a major modifier of the oxygen affinity of hemoglobin (16). However, the regulatory mechanism of PGPase activity in animals is not clear.

In this report, we purified PGPase from *C. reinhardtii*. This is the first homogeneous PGPase purified from a eukaryotic organism. We determined the complete nucleotide sequence of the PGPase cDNA and found characteristics expected to be involved in its regulatory mechanism to help elucidate the physiological importance of PGPase in plants.

EXPERIMENTAL PROCEDURES

Enzyme Purification—All purification steps were carried out at 4 °C unless otherwise specified. *C. reinhardtii* 2137 mt+ cells were grown photoautotrophically under ambient air (aerated with air) at 25 °C as described previously (18) and collected by centrifugation. The cells were disrupted by sonication in 20 mM MES-KOH buffer, pH 6.3, containing 5 mM $MgCl_2$ and 1 mM phenylmethanesulfonyl fluoride and then centrifuged at 150,000 × *g* for 30 min. The supernatant was incubated at 50 °C for 5 min, and the precipitate was removed by centrifugation at 45,000 × *g* for 20 min. The proteins precipitated between 50–60%-saturated $(NH_4)_2SO_4$ were collected and dissolved in 2.5 ml of 20 mM MOPS-KOH buffer, pH 6.3, containing 5 mM $MgCl_2$. After being passed through a Sephadex G-25 column (PD-10, Amersham Pharmacia, Uppsala, Sweden) equilibrated with the same buffer, the fraction was applied to a DEAE-Sephacel column (1 × 7 cm) (Amersham Pharmacia Biotech) equilibrated with the same buffer. Proteins were eluted by changing the buffer to 20 mM Tris-maleate buffer, pH 6.3, containing 5 mM $MgCl_2$. The fractions with PGPase activity were collected and concentrated to approximately 1 ml using a centrifugal concentrator (Ultrafree-15, Millipore, Bedford, MA) and dialyzed overnight against 1

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB052169.

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[1] The abbreviations used are: PGPase, phosphoglycolate phosphatase; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; BT, Blotto-Tween; PMB, *p*-mercuribenzoic acid; EST, expressed sequence tag; NPPase, *p*-nitrophenylphosphatase; DTT, dithiothreitol.

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TABLE I
Purification of phosphoglycolate phosphatase from C. reinhardtii

Purification step	Total protein	Total activity	Specific activity	Purification	Recovery
	mg	*units**	*units mg^{-1} protein*	*-fold*	*%*
Sonicated cell suspension	409	27**	0.066	1.0	100
Crude extract (150,000 × *g* supernatant)	167	27	0.16	2.4	99
50 °C for 5 min	89.5	24	0.27	4.2	89
50–60% sat. $(NH_4)_2SO_4$	22.0	22	0.99	15	81
DEAE-Sephacel	2.15	11	5.3	80	42
Phenyl-Sepharose	0.0664	4.2	64	97	16
Q-Sepharose	0.0168	1.5	87	1300	6

* One unit, 1 μmol Pi min^{-1}.
** Total activity may be underestimated because of the difficulty of assay with the suspension of sonicated cells.

liter of 20 mM MOPS-KOH buffer, pH 7.1, containing 1 mM $MgCl_2$. Solid potassium chloride and EGTA were then added slowly to attain final concentrations of 1.0 M and 1 mM, respectively. After centrifugation (10,000 × *g*, 10 min), the supernatant was applied to a phenyl-Sepharose column (1 × 7 cm) (6FF high sub, Amersham Pharmacia Biotech) equilibrated with 20 mM MOPS-KOH buffer, pH 7.1, containing 1 M potassium chloride, 1 mM $MgCl_2$, and 1 mM EGTA at 25 °C. After the column was washed with 25 ml of the same buffer, proteins were eluted with 20 mM MOPS-KOH buffer, pH 7.1, containing 0.8 M KCl, 1 mM $MgCl_2$, and 1 mM EGTA, and the fractions with PGPase activity were collected. After the addition of Tween 20 (final concentration, 0.001%, v/v), the preparation was concentrated to approximately 0.5 ml using Ultrafree-15 and stored at −20 °C until use. The eluate was loaded onto a Q-Sepharose column (1 × 7 cm) (Amersham Pharmacia Biotech) equilibrated with 20 mM bistrispropane-HCl buffer, pH 7.5, containing 1 mM $MgCl_2$ and eluted with a 75-ml linear gradient of KCl (0–50 mM). Alternatively, the eluate from phenyl-Sepharose was applied to a 5–20% gradient-native PAGE (20). PGPase was detected using activity staining (9) as a white band after the incubation of the gels for 15 min at 25 °C in 20 mM MES-bistrispropane buffer, pH 8.3, containing 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 4 mM phosphoglycolate. The unstained band corresponding to the white band was cut out and homogenized either in 20 mM MOPS-KOH buffer, pH 7.1, containing 5 mM $MgCl_2$ to collect the purified PGPase or in Tris-HCl, pH 6.8, containing 1% SDS, 2% mercaptoethanol, and 0.001% bromphenol blue to apply SDS-PAGE using a 12.5% polyacrylamide slab gel (20).

Determination of the N-terminal Sequence—The separated proteins on SDS-PAGE gel were transferred onto a polyvinylidene difluoride membrane using a semidry transfer apparatus (TransBlot SD, Bio-Rad) after washing the gel with water for 5 min. After staining the membrane with 0.025% Coomassie Brilliant Blue R-250 in 40% methanol, the peptide band was excised and subjected to determination of N-terminal amino acid sequence by Edman degradation using an Applied Biosystems Model 477A sequencing system (Applied Biosystems, Foster City, CA).

DNA Sequence—DNA were sequenced by using the BigDye Terminator DNA Sequencing Kit and an ABI PRISM 377 DNA sequencer (Applied Biosystems).

Transformation of Chlamydomonas Cells—C. reindardtii was transformed with plasmid DNA by electroporation (21) with the slight modification of HS medium being replaced with min-70 medium (4).

Immunoblotting—Proteins in the crude extract were separated on SDS-PAGE gels and transferred onto polyvinylidene difluoride membrane as described above. The blots were blocked overnight in a Tris-buffered saline buffer containing 5.5% (w/v) nonfat skim milk, 2% bovine serum albumin, 0.1% (v/v) Tween 20, and 0.02% (w/v) sodium azide at 4 °C, washed with Tris-buffered saline containing 0.1% Tween 20 and 5.5% nonfat skim milk (BT), and incubated with the primary antibody for 180 min. After being washed four times with BT, the membrane was incubated for 60 min with anti-rabbit IgG biotin conjugate (1:1000 dilution in BT, Sigma). After being washed three times with BT and 1 time with Tris-buffered saline containing 0.1% Tween 20, the membrane was incubated with streptavidin-horseradish peroxidase conjugate (1:1000 dilution in Tris-buffered saline containing 0.1% Tween 20, Life Technologies, Inc.). After being washed five times with Tris-buffered saline containing 0.1% Tween 20 and two times with 20 mM Tris-HCl, pH 7.5, the peptides that reacted with the primary antibody were visualized in 20 mM Tris-HCl, pH 7.5, containing 0.025% 3.3′-diaminobenzidine tetrahydrochloride, 0.03% hydrogen peroxide, and 0.3% $CoCl_2$.

Preparation of the Primary Antibody—A synthetic peptide AS1787, corresponding to the N-terminal 17-amino acid sequence of purified PGPase subunit with cysteine substituted for the N-terminal Ser, was generated by Takara Shuzo Co., Ltd. (Kusatsu, Japan). The antibody against AS1787 was generated and purified by using rProtein A-Sepharose FF (Amersham Pharmacia Biotech) and CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) coupled with AS1787 by the same company.

Estimation of Molecular Mass—The PGPase preparation after DEAE-Sephacel was applied to the HiLoad 16/60 Superdex 200-pg column (Amersham Pharmacia Biotech) equilibrated with 20 mM MOPS-KOH, pH 8.0, 5 mM $MgCl_2$, 100 mM KCl, and 1 mM EDTA at the rate of 1 ml min^{-1} for the molecular mass determination.

Isoelectric Focusing—Isoelectric focusing analysis of PGPase was carried out in 6.5% (w/v) polyacrylamide gel (22) using Anpholine® preblended pH 3.5–9.5 for isoelectric focusing analysis (Amersham Pharmacia Biotech) and the isoelectric focusing markers (Isoelectric Focusing Mix 3.6–9.3, Sigma). PGPase was detected by the activity-staining method described above.

Enzyme Assay and Protein Determination—PGPase activity was determined by measuring the phosphoglycolate-dependent release of inorganic phosphate (23) as described previously using the assay mixture containing 20 mM MES-bistrispropane, pH 8.0, 5 mM $MgCl_2$, and 4 mM phosphoglycolate (18). Protein was quantified by the method of Bradford (24) using bovine serum albumin as a standard.

Chlorophyll Content Determination—Chlorophyll content was determined after extraction with 96% (v/v) ethanol (25).

RESULTS

Purification of PGPase—Table I summarizes the purification steps for PGPase from *C. reinhardtii*. PGPase eluted from the DEAE-Sephacel column by changing 20 mM MOPS-KOH in the buffer to 20 mM Tris-maleate had a higher specific activity than that of the final preparation reported by Husic and Tolbert (14). The next step involving phenyl-Sepharose column chromatography was most effective in attaining a specific activity comparable to that from higher plants (Table I), although PGPase was still not the major protein after this step (Fig. 1, *lane 1*). PGPase activity was stable after the phenyl-Sepharose step for at least 6 months at −20 °C. The single peptide band with a molecular mass of 32 kDa was obtained by SDS-PAGE after the next purification step, either Q-Sepharose column chromatography or native PAGE (Fig. 1). The purified PGPase after Q-Sepharose chromatography had a specific activity of 86.5 μmol P_i (mg protein)$^{-1}$ min^{-1}, a 1300-fold increase from that of the sonicated cell suspension (Table I).

Molecular Mass, pI Value, K_m Value, and Substrate Specificity—The molecular mass was estimated to be ~65 kDa for the native PGPase by Superdex 200 (Fig. 2) and ~32 kDa for the subunit by SDS-PAGE (Fig. 1), suggesting PGPase to be a homodimer. The isoelectric point was estimated to be 5.1 (data not shown). The K_m value for phosphoglycolate was ~140 μM (see Fig. 9). Consistent with the partially purified PGPases from higher plants (9, 11), the purified PGPase from *C. reinhardtii* was also highly specific for phosphoglycolate. After the Q-Sepharose step, the enzyme did not at all hydrolyze 3-phosphoglycerate, D-ribose-5-phosphate, phosphoenolpyruvate, *O*-phospho-L-serine, or *p*-nitrophenylphosphate, although it hydrolyzed ribulose-5-phosphate and fructose-1,6-bisphosphate

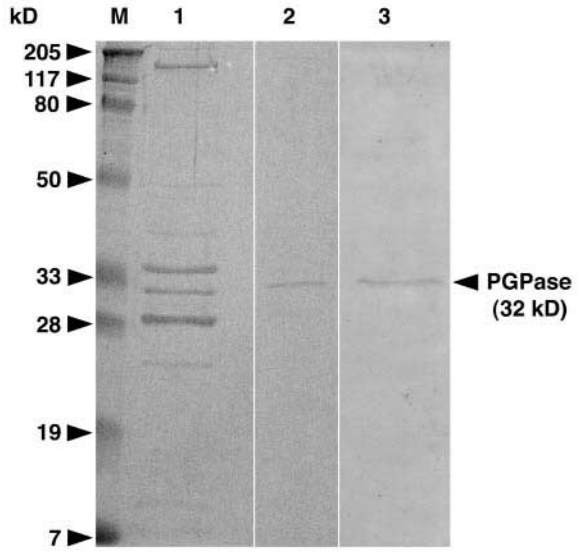

FIG. 1. **SDS-PAGE analysis of the purified PGPase from *C. reinhardtii* strain 2137.** *Lane 1*, PGPase fraction eluted from phenyl-Sepharose; *lane 2,* PGPase fraction eluted from Q-Sepharose after phenyl-Sepharose chromatography; *lane 3,* the band with PGPase activity after native PAGE; *lane M,* molecular markers (prestained SDS-PAGE standards, Broad Range, Control #76943, Bio-Rad). *Numbers* at the *left* denote molecular mass. Proteins in the gel were visualized by Quick-CBB (Wako Chemicals, Osaka, Japan) for *lanes 1* and *2* and by silver staining for *lane 3*.

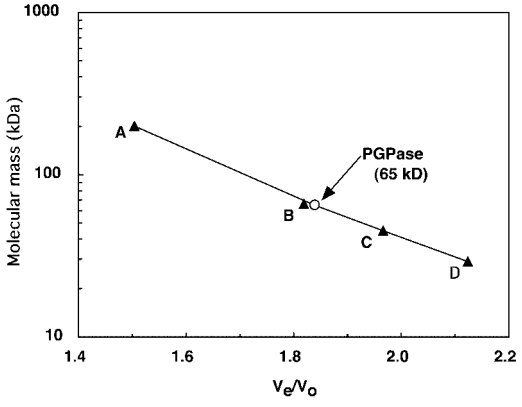

FIG. 2. **Determination of the molecular mass of native PGPase purified from *C. reinhardtii* strain 2137.** The molecular markers used for the calibration were β-amylase (*A*, 200 kDa), bovine serum albumin (*B*, 66 kDa), ovalubmin (*C*, 45 kDa), and carbonic anhydrase (*D*, 29 kDa).

at a rate of 1–4% of that of phosphoglycolate hydrolysis (data not shown).

Amino Acid Sequence—The N-terminal amino acid sequence of the purified PGPase was determined up to 21 residues, SARPIATNEQKLELLKKVESF. By searching the expressed sequence tag (EST) data base for *C. reinhardtii* strain C9 (26), we found an EST encoding the 21-residues amino acid sequence of PGPase with the except that the 20th residue was a cysteine, which was hardly distinguishable from serine by the amino acid sequencer. The complete nucleotide sequence (GenBank™ accession number AB052169) of the EST clone contained an open reading frame encoding a polypeptide consisting of 330 amino acids. The deduced amino acid sequence of the EST clone was similar to that of *p*-nitrophenylphosphatase (NPPase) in yeast and the relatives in various other organisms (Fig. 3). A close similarity was observed especially to an NPPase-like protein in *A. thaliana* with a 52% identity for the entire sequence (Fig. 3) and 72% for the middle 250 residues. *C. reinhardtii* PGPase also exhibited a high similarity to the ESTs from various higher plants, such as cotton (GenBank™ accession number AI055133), *Lotus japonicus* (GenBank™ accession number AV423957), rice (GenBank™ accession number AA751972), soybean (GenBank™ accession number AW755938), tomato (GenBank™ accession number AI777250), pine (GenBank™ accession number AW056729), and wheat (GenBank™ accession number BE425843). On the other hand, *C. reinhardtii* PGPase was not so similar to prokaryote PGPases with <30% identity to those from *Alcaligenes eutrophus* (GenBank™ accession number M68904), *Aquifex aeolicus* (GenBank™ accession number AE000735), *Escherichia coli* (GenBank™ accession number AE000414), *Synechocystis* sp. strain PCC6803 (GenBank™ accession number D90907). However, two sequence motifs, FD*X*DG and VGD (Fig. 3), were well conserved among PGPases in prokaryotes at the similar positions (data not shown).

The first 28 N-terminal amino acid residues, which were not contained in the purified PGPase protein, have the characteristics of a transit peptide of *C. reinhardtii*. The amino acid sequence was rich in Arg and Ala and contained no acidic residue. Four Arg residues spaced by two or three neutral amino acids in the middle region were just within the random coil region between two amphiphilic helical motifs consisting of five or six residues (Fig. 4). The amino acid sequence just upstream of the N-terminal of the purified enzyme, VAAQA, was similar to the motif V*X*A (27). These characteristics are consistent with the typical features of a stroma transit peptide (27, 28), indicating that PGPase in *C. reinhardtii* is a stroma protein (14) as reported in higher plants (11). The predicted molecular mass and isoelectric point of the mature subunit of 302 amino acids were approximately 33kDa and 5.6, respectively, consistent with those determined for the purified PGPase as described above.

Although 425 proteins containing the motif FD*X*DG were found in the Swiss-Prot data base at the ExPASy site (www.expasy.org), they were mainly Ca^{2+}-binding or phosphate-related proteins. The amino acid sequence of the PGPase contained two regions similar to EF-hand motifs of Ca^{2+}-binding proteins such as *C. reinhardtii* calmodulin (Fig. 4), although three residues did not match in both regions to the consensus pattern of EF-hand: D*X*-[DNS]-{ILVFYW}-[DENSTG]-[DNQGHRK]-{GP}-[LIVMC]-[DENQSTAGC]-X^2-[DE]-[LIVMFYW].

Immunodetection of PGPase Subunit—The affinity-purified antibody raised against a synthetic peptide AS1787 (see under "Experimental Procedures") reacted strongly with the subunit of purified PGPase in the immunoblot analysis (Fig. 5, *lane 6*). Several other bands were also detected by the antibody in crude extract of wild-type 2137 (*lanes 8*-10), but they were also detected by the preimmune serum (*lanes 3–5*). The 32-kDa peptide corresponding to the PGPase subunit seems to be the only band detected specifically by the antibody and was not detected in a PGPase-deficient mutant *pgp1*-18–7F, in which the activity to hydrolyze *p*-glycolate was marginal (3, 17, 18) and was not caused by the PGPase with the optimal pH of approximately 8 (18). These findings suggest that the PGPase we purified is the PGPase essential for growth under ambient air from which activity is missing in the mutant. It was also reported that PGPase activity in 2137 increased by ~60% 5 h after transfer 5% CO_2-grown cells to air (17). However, not so much difference was observed between the immunoblot profiles of 5% CO_2-grown and 5-h air-adapting cells of 2137, although PGPase activity increased by ~50% (Fig. 5).

Complementation of pgp1 Mutation—Four clones in a genomic DNA library of *C. reinhardtii* C9 (21) showed strong signals in two separate Southern blotting experiments probed with independent digoxigenin-labeled polymerase chain reac-

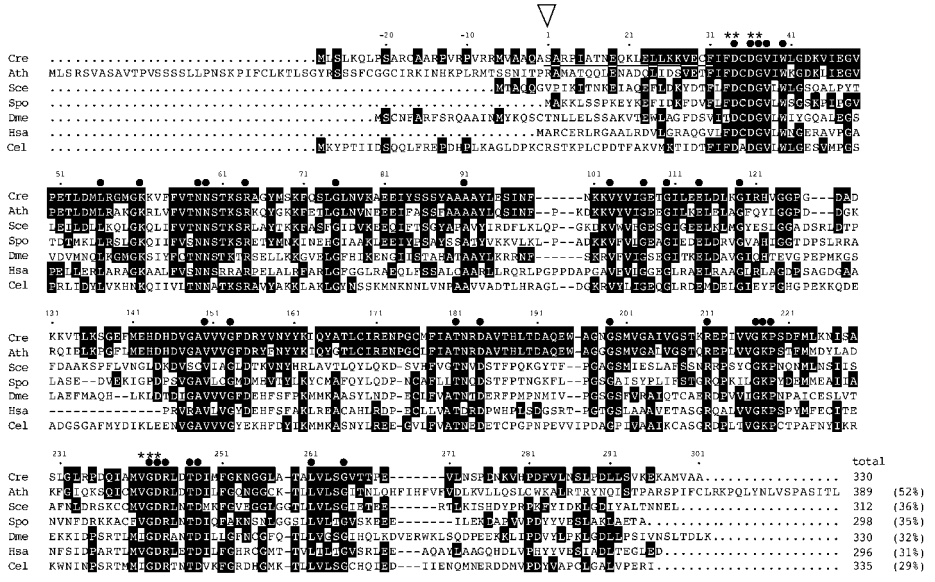

FIG. 3. **Alignment of the deduced amino acid sequences of the *C. reinhardtii PGP1* gene product and some NPPase-like gene products from various organisms.** Amino acid residues identical to those of the *C. reinhardtii PGP1* (PGPase precursor) are *boxed* in *black*. *Closed circles*, residues conserved in all sequences compared; *, residues conserved well in the prokaryote PGPases; *open triangle*, the N-terminal Serine residue of the mature PGPase subunit. The region corresponding to N-terminal sequence of the purified PGPase is *underlined*. *Cre*, *C. reinhardtii* PGPase precursor (GenBank™ accession number AB52169); *Ath*, *A. thaliana* NPPase-like protein BAA98057.1 (GenBank™ accession number AB025605); *Sce*, *Saccharomyces cerevisiae* NPPase (GenBank™ accession number X51611); *Spo*, *Schizosaccharomyces pombe* NPPase (GenBank™ accession number AL031349); *Dme*, *Drosophila melanogaster* CG5567 gene product AAF49296.1 (GenBank™ accession number AE003522); *Hsa*, *Homo sapiens* protein dj37E16.5 (GenBank™ accession number Z83844); *Cel*, *Caenorhabditis elegans* hypothetical protein AAC25793.1 (GenBank™ accession number AF016421).

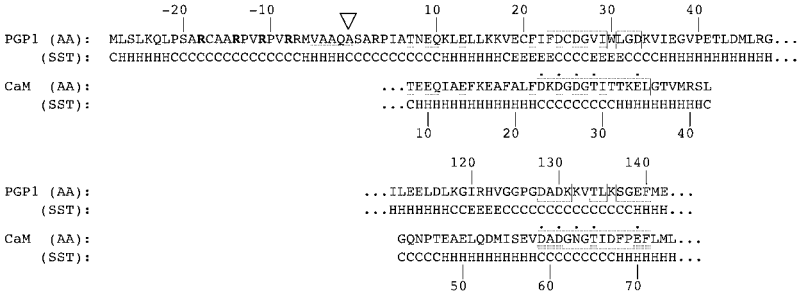

FIG. 4. **Comparison of the deduced amino acid sequence with predicted secondary structure of PGPase precursor from *C. reinhardtii*.** *PGP1*, PGPase precursor; *CaM*, calmodulin from *C. reinhardtii* (Swiss-Prot accession number P04352); (*AA*), amino acid sequence; (*SST*), secondary structure predicted by SSTread (39) at the DDBJ site (www.ddbj.nig.ac.jp). Arg residues matching well with the characteristics of stroma transit peptides are in *boldface*. A motif similar to V*X*A that is often found just before the processing site of stroma-targeting proteins is *underlined*. *Closed triangle*, the processing site suggested by N-terminal sequence of purified PGPase. The residues matching between PGP1 and *C. reinhardtii* CaM are *double underlined*. *Boxed* residues match with the consensus motif in EF-hand region of Ca^{2+}-binding proteins; *H,* residues predicted to form an α-helix; *E,* residues predicted to form β-strand; *C,* residues predicted to form random-coil structure.

tion products of the genomic DNA from a wild-type 2137 by using primers designed based on the cDNA sequence of PGP1 (data not shown). The DNA fragments from these clones were used for the transformation (21) of N142, a wall-less progeny of the *pgp1* mutant after crossing 18-7F with *cw15* strains. The genomic clones 57B9 and 60C10 successfully restored the growth of the *pgp1* mutant under air. In a transformant with 57B9, the growth rate under air was approximately 40% of that of wild type, and PGPase activity was approximately 25% of that of wild type. These results support that the high CO_2-requiring phenotype of *pgp1* is caused by the deficiency in PGPase activity, and that the sequenced cDNA represents a physiologically functional PGPase.

mRNA Levels During the Adaptation to Air—We observed an increase in mRNA level of PGPase 5 h after transferring the wild-type cells from 5% CO_2 to air (Fig. 6). Although there was not so much increase in the peptide level of PGPase (Fig. 5), the increase in the activity of PGPase 5 h after transfer to air is probably expected to be a result of a posttranscriptional regulation.

Optimal pH—Husic and Tolbert (14) reported a broad pH optimum with the maximal activity approximately pH 6.3 for partially purified PGPase from *C. reinhardtii*. However, this profile was uncertain, because they used a very complicated buffer system using 20 mM acetate, ~pH 6.3. We observed a 50% inhibition of PGPase activity by 20 mM acetate (data not shown). We determined the pH profile using 20 mM 3,3-dimethylglutarate-KOH as the buffer for acidic pH and 20 mM MES-bistrispropane for basic pH (Fig. 7). The optimal pH was between 8 and 9, and the activity at pH 6.3 was less than half that at pH 8 in the enzyme purified with DEAE-Sephacel, which was quite similar to that reported in *H. cylindracea* (13), although the activity was relatively higher between pH 5 and 7 in the crude extract (Fig. 7).

Effect of Calcium—It was reported that PGPase from maize was inhibited very strongly by Ca^{2+}, and this was reversed by Mg^{2+} (11). We also observed the reversible inhibition in the purified PGPase from *C. reinhardtii* (data not shown). The inhibition increased with decreasing concentration of Mg^{2+} in the reaction mixture (Fig. 8), and the IC_{50} value for $CaCl_2$ was ~50

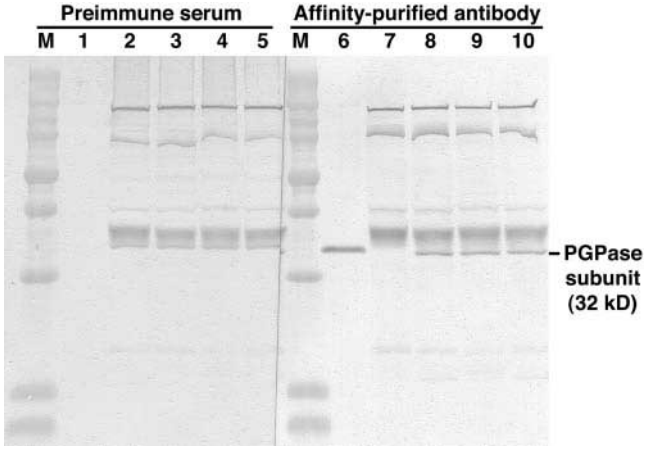

FIG. 5. **Immunoblotting analysis of PGPase before and after 5-h adaptation of 5% CO_2-grown cells to air.** *C. reinhardtii* wild-type 2137 cells grown under 5% CO_2 in six flasks containing 250 ml of min-74 liquid medium were combined, mixed, and distributed into six flasks again. Three were used for the RNA analysis (see Fig. 6), and the other three were exposed to three different conditions, 0 h in air (350 ppm of CO_2), 5 h in air, and 5 h in 5% CO_2. *Lanes 1* and *6*, purified PGPase; *lanes 2* and *7*, 5% CO_2-grown cells of PGPase-deficient mutant *pgp1*-18–7F; *lanes 3* and *8*, 5% CO_2-grown cells of 2137 (0-h air cells); *lanes 4* and *9*, 5 h of air-adapting cells of 2137 (5-h air cells); *lanes 5* and *10*, 5% CO_2-grown cells of 2137 (5-h CO_2 cells); *lane M*, molecular markers (precision protein standards, Control 89944, Bio-Rad). *Lanes 1–5,* probed with the preimmune serum; *lanes 7–10*, probed with the affinity-purified antibody raised against a synthetic peptide AS1787. PGPase activities of 0-h air, 5-h air, and 5-h CO_2 cells of 2137 were 29, 42, and 28 μmol^{-1} P_i release·mg Chl·h^{-1}, respectively.

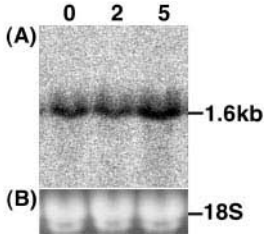

FIG. 6. **RNA gel blot analysis of PGPase in *C. reinhardtii* strain 2137 before and after transferring 5% CO_2-grown cells to air.** Total RNA was isolated and analyzed as described previously (40) using the cells in three flasks prepared as described in Fig. 5. *A,* RNA gel blot showing the level of PGP1 mRNA after 0, 2, and 5 h after transfer to air (350 ppm of CO_2). *B,* 18 S rRNA stained with ethidium bromide shown as a loading control.

and 175 μM with 0.5 and 1 mM $MgCl_2$, respectively (data not shown). The addition of 5 mM EGTA also resulted in 100% recovery after inhibition by $CaCl_2$, whereas EGTA did not affect the activity of PGPase in the absence of $CaCl_2$ (Table II) even after preincubation with 5 mM EGTA (data not shown). Fig. 9 compares the double reciprocal plots of PGPase activity at different concentrations of $CaCl_2$. The affinity for phosphoglycolate was not affected by Ca^{2+}, indicating it to be a non-competitive inhibition with respect to its substrate. The subunit of PGPase pretreated with Ca^{2+} migrated faster in SDS-PAGE than PGPase pretreated only with EGTA (Fig. 10), which is characteristic of Ca^{2+}-binding proteins (29).

Effect of Ribose-5-Phosphate—Ribose-5-phosphate has been reported to be a competitive inhibitor of spinach PGPase (8, 10). However, unlike in spinach, ribose-5-phospate did not inhibit but rather activated *C. reinhardtii* PGPase (Fig. 11).

Effect of Other Compounds—Table II shows the effect of some other compounds on the activity of purified PGPase from *C. reinhardtii*. The activity was strongly inhibited by PMB with an IC_{50} of 0.45 μM, although only a slow inhibition by *N*-ethylmaleimide was reported in tobacco PGPase (30). The activity recovered ~90% following the addition of 10 mM DTT, whereas 10 mM DTT did not affect the activity without PMB treatment even after 2-h treatment at 30 °C. These results suggest that at least one of the SH groups of four Cys residues (Fig. 3) has a potential to modulate the activity. Treatment with 10 mM DTT at 30 °C for 2 h did not change the mobility of PGPase in the native PAGE (data not shown). Mobility was not changed in SDS-PAGE either with or without 2-mercaptoethanol (data not shown). These results indicate that there is no S–S bond between the subunits. Activity was inhibited by *O*-phenanthroline, a metal complex reagent, but the IC_{50} value of 1 mM was higher than in ordinary metaloenzymes. PGPase activity was not sensitive to sodium azide, suggesting that the enzyme does not contain heme.

DISCUSSION

PGPase is essential for all autotrophic organisms and is also important for the function of human red blood cells (16). However, there is little information about its regulation and no information about the molecular structure of eukaryotic PGPases. Here we present the first report on the purification of a eukaryotic PGPase with high enough purity to enable the determination of its N-terminal amino acid sequence of up to 21 residues, which allowed us to determine the complete nucleotide sequence of cDNA.

We concluded that PGPase from *C. reinhardtii* is a homodimer with a molecular mass of ~65 kDa consisting of two identical 32-kDa subunits whose molecular masses were predicted to be 33 kDa from the cDNA sequence. Eukaryotic PGPases were reported to be homodimers with almost the same native molecular mass among a wide variety of organisms, such as maize (11), tobacco (9), spinach (31), and human (15). Earlier reports proposed a much higher molecular mass of PGPases from plants and algae, 92 kDa as the native molecular mass for the partially purified enzyme from *C. reinhardtii* (14), 93 kDa for spinach, and 81–86 kDa (tetramer) for tobacco (30). However, such values were shown to be quite unlikely, at least in spinach and tobacco (9), and the inconsistency may be partly a consequence of the insufficient purity in those PGPase preparations. The pI value of 5.1 for the purified PGPase from *C. reinhardtii* was consistent with those reported among a wide variety of organisms, 4.9 for PGPase from maize leaves (11), 4.4 for PGPase from pea (12), 4.2 and 5.5 for two isoforms from french bean (32), and 5.0 for PGPase from human red blood cells (15). On the other hand, the reported kinetic characteristics of PGPase vary widely among the different species. The K_m value for phosphoglycolate was reported to be 26 μM in spinach (10) and tobacco (30), 222 μM in the cyanobacteria *C. peniocystis* (7), 570 μM in maize (11), and 800 μM in the brown algae *H. cylindracea* (13). Much larger values of 2 mM have been reported in pea (12) and bean (32). The K_m value of 140 μM determined for *C. reinhardtii* in our study was similar to that of *C. peniocystis*, intermediate to that of spinach and maize, although the value of 23 μM reported earlier for *C. reinhardtii* (14) was as small as that in spinach and tobacco. Such diversity may be a result of some differences in experimental conditions, because the values can be affected by the concentrations of inorganic phosphate, ribose-5-phosphate, and $MgCl_2$ (8, 16, 33).

Ca^{2+} may have an important role in the regulation of PGPase activity *in vivo*. Our results indicated that *C. reinhardtii* PGPase was a Ca^{2+}-binding protein and was strongly but reversibly inhibited by Ca^{2+}, although the effective concentration appeared higher than that usually observed in cells. It is very probable that at least the first EF-hand-like region in *C. reinhardtii* PGPase (Fig. 4) is responsible for Ca^{2+} binding. The region was more similar than the second one to the EF-hand motif and was observed at almost the same position from the

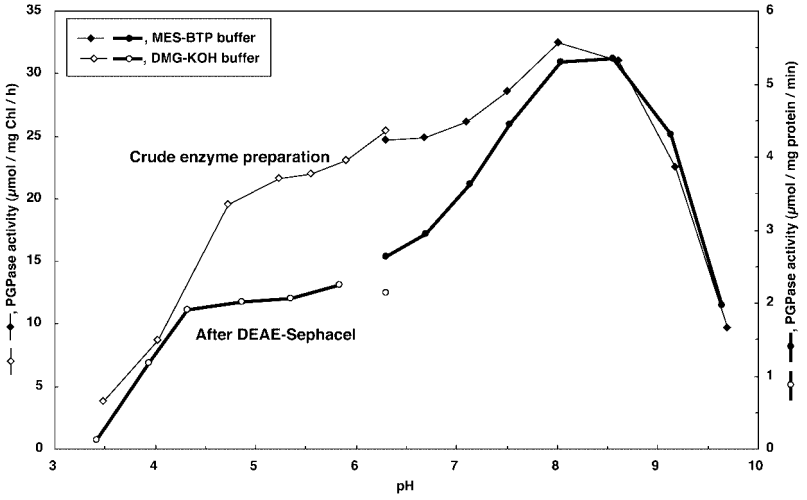

FIG. 7. **Effect of pH on the activity of PGPase from *C. reinhardtii* strain 2137.** The pH profiles of the enzyme preparation after DEAE-Sephacel and the crude enzyme preparation were compared. The activity in the crude preparation represents the sum of activities in both supernatant and precipitate after sonicated cells were centrifuged at 150,000 × *g* (18).

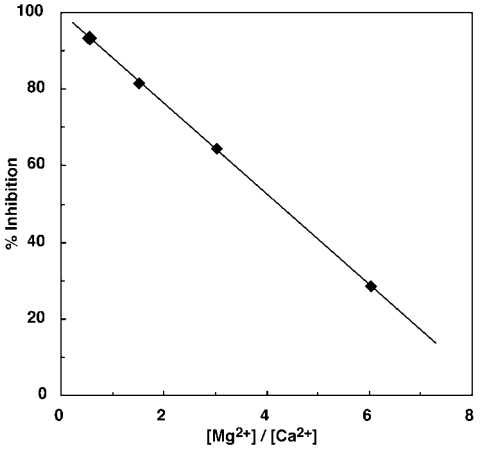

FIG. 8. **Effect of $MgCl_2$ and $CaCl_2$ concentration on PGPase activity.** The activity was determined in the preparation after phenyl-Sepharose chromatography using our standard method with the exception of the preincubation period of $MgCl_2$ and $CaCl_2$ concentrations. The concentrations of $MgCl_2$ and $CaCl_2$ were 0.83 and 1.66 mM, 5.0 and 3.38 mM, 5.0 and 1.66 mM, and 5.0 and 0.83 mM, respectively.

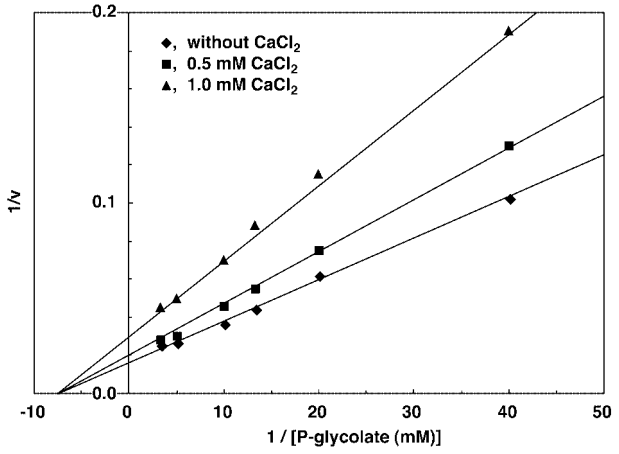

FIG. 9. **Effect of $CaCl_2$ on the double reciprocal plot of PGPase activity *versus* phosphoglycolate concentration.** The activity in the enzyme preparation after phenyl-Sepharose chromatography (0.18 μg of protein) was assayed using our standard method with the exception of phosphoglycolate and $CaCl_2$ concentrations.

TABLE II
Effect of some compounds on the activity of PGPase from C. reinhardtii

Compound	Concentration	Activity
	M	*% control*
PMB	4.5×10^{-7}	50
	1.0×10^{-6}	0
(+ 10 mM DTT)*	1.0×10^{-6}	90
DTT**	1.0×10^{-2}	100
O-phenanthroline	5.0×10^{-4}	100
	8.0×10^{-4}	70
	1.0×10^{-3}	50
NaN_3	3.0×10^{-2}	100
EGTA	1.0×10^{-2}	99
EDTA	1.0×10^{-2}	63

* DTT was added after 100% inhibition by PMB was confirmed.
** The activity was measured after preincubation at 30 °C for 1 h.



FIG. 10. **Effect of $CaCl_2$ on the mobility of the PGPase subunit in SDS-PAGE.** The enzyme preparation after phenyl-Sepharose chromatography was denatured with the sample buffer without $CaCl_2$ (*lanes 1* and *3*) or with 50 mM $CaCl_2$ (*lanes 2* and *4*). Protein amount loaded was 2 μg (*lanes 1* and *2*) and 1 μg (*lanes 2* and *4*). PGPase subunit was visualized by immunoblotting as shown in Fig. 5.

N-terminal to that of the first EF-hand of calmodulins. However, the main role of this region is more likely to bind Mg^{2+}, because Ca^{2+} was a competitive inhibitor with respect to Mg^{2+}. Although PGPase requires a divalent cation such as Mg^{2+} and a monovalent anion such as Cl^- for activity, the enzyme is probably saturated with these activators *in vivo* (7). However, it is still possible that the activity is regulated by the Mg^{2+}/Ca^{2+} ratio. On the other hand, it was proposed that maize PGPase was regulated by light through both energy charge and NADP(H) via a regulatory site(s) without modification of the protein by the ferredoxin-thioredoxin system (19). *C. reinhardtii* PGPase may also be regulated in a similar way, as we did not detect any effect of DTT on the activity in the absence of the SH-reagent. It is still possible, however, that PGPase activity is regulated by the modification of Cys residues. The addition of DTT resulted in the recover of the activity after complete inactivation by PMB, and the two most N-terminal Cys were arranged as a C*XXXX*C motif, which is also observed in some thioredoxin-targeted stroma enzymes as the regulatory site (34). The corresponding C*XXXX*C motif was not found among other NPPase-like gene products (Fig. 3), although Cys corresponding to the C-terminal end of the motif was conserved well. It should also be pointed out that two other stroma phosphatases, fructose-1,6-bisphosphatase, and sedoheptulose-1,7-bisphosphatase are thioredoxin-targeted enzymes with the

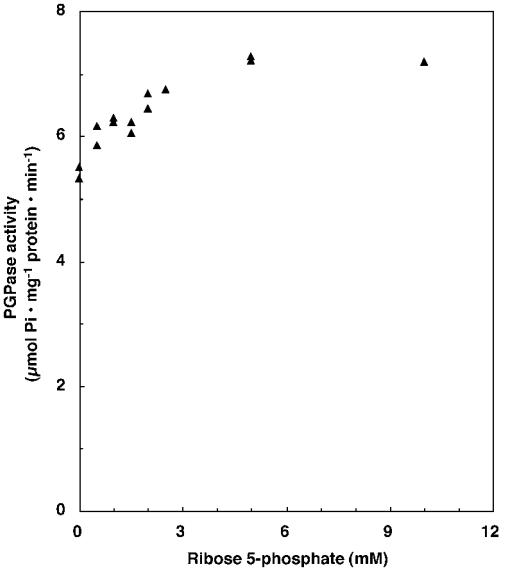

FIG. 11. **Effect of ribose-5-phosphate on the activity of PGPase purified from *C. reinhardtii*.** The activity in the preparation after phenyl-Sepharose chromatography was assayed using our standard method with the exception that phosphoglycolate concentration was 750 μM.

C*XXXX*C motif (34) and are also known to be inhibited by Ca^{2+} (35–37).

It is still unclear how PGPase activity increases by 50–60% within 5 h when 5% CO_2-grown cells were transferred to air. The activity does not seem to be regulated by the change in the amount of enzyme (Fig. 6). PGPase activity may be regulated during the adaptation by the change in one or some of the possible metabolites, such as phosphoglycolate, ribose 5-phosphate, and inorganic phosphate (30), and/or the change(s) in Mg^{2+}/Ca^{2+} ratio, energy charge, pH, NADP(H), thioredoxins, or glutathione in the stroma.

Deduced amino acid sequence of *C. reinhardtii* PGPase exhibited significant homology to NPPase-like gene products in yeast and animals. Together with close similarities in molecular properties, such as the molecular mass, pI value, and the homodimeric composition discussed above, this finding suggests that some of the NPPase-like gene products in these organisms are also PGPases, althouth dj37E16.5 (Fig. 3) may not be a human PGPase, because the gene is located in chromosome 22. Instead, we found the sequence more similar to that of *C. reinhardtii* PGPase in a working draft sequence (GenBank™ accession number AC012171) from chromosome 16 in which the human PGPase gene is located (38). The NPPase-like gene product from *A. thaliana* is probably PGPase rather than NPPase based on the close similarity in the deduced amino acid sequence to that of *C. reinhardtii* PGPase. Similarly, ESTs from several other plant species are also good candidates for the genes encoding PGPase. These results suggest that eukaryotic PGPases in various organisms ranging from animals to plants share a common ancestral molecule as proposed by Rose *et al.* (16). Although they were not similar to prokaryotic PGPases in both structure and function, our knowledge on PGPases only came from quite a limited number of organisms. Thus, a more extensive comparison is necessary on the structure and function of PGPases in both eukaryotes and prokaryotes to dissolve this missing link.

In this study, it was also shown that the PGPase protein we purified is missing in the PGPase-deficient mutant *pgp1*-18–7F, whereas the nuclear recessive mutation in this mutant is still not clear in the molecular level (3, 17, 18). Further studies are in progress for this mutant.

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REFERENCES

1. Somerville, C. R., and Ogren W. L. (1979) *Nature* **280,** 833–836
2. Hall, N. P., Kendall, A. C., Lea, P. J., Turner, J. C., and Wallsgrove, R. M. (1987) *Photosynth. Res.* **11,** 89–96
3. Suzuki, K., Marek, L. F., and Spalding M. H. (1990) *Plant Physiol. (Bethesda)* **93,** 231–237
4. Suzuki, K., Mamedov, T. G., and Ikawa, T. (1999) *Plant Cell Physiol.* **40,** 792–799
5. Wolfenden, R. (1970) *Biochemistry* **9,** 3404–3407
6. Anderson, L. (1971) *Biochim. Biophys. Acta* **235,** 237–244
7. Norman, E. G., and Colman, B. (1991) *Plant Physiol. (Bethesda)* **95,** 693–698
8. Christeller, J. T., and Tolbert, N. E. (1978) *J. Biol. Chem.* **253,** 1780–1785
9. Belanger, F. C., and Ogren, W. L. (1987) *Photosynth. Res.* **14,** 3–13
10. Husic, H. D., and Tolbert, N. E. (1984) *Arch. Biochem. Biophys.* **229,** 64–72
11. Hardy, P., and Baldy, P. (1986) *Planta* **168,** 245–252
12. Kerr, M. W., and Gear, C. F. (1974) *Biochem. Soc. Trans.* **2,** 338–340
13. Randall, D. D. (1976) *Aust. J. Plant Physiol.* **3,** 105–111
14. Husic, H. D., and Tolbert, N. E. (1985) *Plant Physiol. (Bethesda)* **79,** 394–399
15. Zecher, R., Schwuléra, U., and Wolf, H. U. (1982) *Int. J. Biochem.* **14,** 775–781
16. Rose, Z. B., Grove, D. S., and Seal, S. N. (1986) *J. Biol. Chem.* **261,** 10996–11002
17. Marek, L. F., and Spalding, M. H. (1991) *Plant Physiol. (Bethesda)* **97,** 420–425
18. Suzuki, K. (1995) *Plant Cell Physiol.* **36,** 95–100
19. Baldy, P., Jacquot, J.-P., Lavergne, D., and Champigny, M. L. (1989) *Photosynth. Res.* **22,** 147–155
20. Laemmli, U. K. (1970) *Nature* **227,** 680–685
21. Fukuzawa, H., Miura, K., Ishizaki, K., Kucho, K., Saito, T., Kohinata, T., and Ohyama, K. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 5347–5352
22. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250,** 4007–4021
23. Ames, B. N. (1966) *Methods Enzymol.* **8,** 115–118
24. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
25. Wintermans, J. F. G. M., and De Mots, A. (1965) *Biochim. Biophys. Acta* **109,** 448–453
26. Asamizu, E., Nakamura, Y., Sato, S., Fukuzawa, H., and Tabata, S. (1999) *DNA Res.* **6,** 369–373
27. Franzén, L.-G., Rochaix, J.-D., and Heijne, G. V. (1990) *FEBS Lett.* **260,** 165–168
28. Krimm, I., Gans, P., Hernadez, J.-F., Arlaud, G. J., and Lancelin, J.-M. (1999) *Eur. J. Biochem.* **265,** 171–180
29. Roberts, D. M., and Harmon, A. C. (1992) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43,** 375–414
30. Christeller, J. T., and Tolbert, N. E. (1978) *J. Biol. Chem.* **253,** 1791–1798
31. Seal, S. N., and Rose, Z. B. (1987) *J. Biol. Chem.* **262,** 13496–13500
32. Verin-Vergeau, C., Baldy P., and Cavalie G. (1979) *Phytochemistry* **18,** 1279–1282
33. Christeller, J. T., and Tolbert, N. E. (1978) *J. Biol. Chem.* **253,** 1786–1790
34. Schürmann, P., and Jacquot, J.-P. (2000) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **51,** 371–400
35. Charles, S. A., and Halliwell, B. (1980) *Biochem. J.* **188,** 775–779
36. Hertig, C., and Wolosiuk, R. A. (1980) *Biochem. Biophys. Res. Commun.* **97,** 325–333
37. Wolosiuk, R. A., Hertig, C. M., and Busconi, L. (1982) *FEBS Lett.* **140,** 31–35
38. Mulley, J. C., Barton, N., and Callen, D. F. (1990) *Cytogenet. Cell Genet.* **53,** 175–176
39. Ito, M., Matsuo, Y., and Nishikawa, K. (1997) *Comput. Appl. Biosci.* **13,** 415–423
40. Kucho, K., Ohyama, K., and Fukuzawa, H. (1999) *Plant Physiol. (Bethesda)* **121,** 1329–1337