Chlamydomonas reinhardtii phosphoglycerate kinase (PGK1) from the eukaryotic green alga characterized the redox features of the Calvin-Benson enzyme. The Calvin-Benson cycle may withstand redox regulation. Here, we approached four enzymes of the chloroplastic thioredoxin. Recently, proteomics-based approaches suggested that not only four but all enzymes of the chloroplastic thioredoxin. Recently, several proteomics-based approaches suggested that not only four but all enzymes of the Calvin-Benson cycle may withstand redox regulation. Here, we characterized the redox features of the Calvin-Benson enzyme phosphoglycerate kinase (PGK1) from the eukaryotic green alga Chlamydomonas reinhardtii, and we show that C. reinhardtii PGK1 (CrPGK1) activity is inhibited by the formation of a single regulatory disulfide bond with a midpoint redox potential (−335 mV at pH 7.9). CrPGK1 oxidation was found to affect the turnover number without altering the affinity for substrates, whereas the enzyme activation appeared to be specifically controlled by f-type thioredoxin. Using a combination of site-directed mutagenesis, thiol titration, mass spectrometry analyses, and three-dimensional modeling, the regulatory disulfide bond was shown to involve the not strictly conserved Cys227 and Cys361. Based on molecular mechanics calculation, the formation of the disulfide is proposed to impose structural constraints in the C-terminal domain of the enzyme that may lower its catalytic efficiency. It is therefore concluded that CrPGK1 might constitute an additional light-modulated Calvin-Benson cycle enzyme with a low activity in the dark and a TRX-dependent activation in the light. These results are also discussed from an evolutionary point of view.

The importance of regulatory mechanisms involving dithiol/disulfide exchange reactions has been thoroughly investigated in most organisms. The oxidoreduction of disulfide bonds is a reversible redox modification that can modulate the activity of numerous proteins and is largely controlled by a class of small oxidoreductases named thioredoxins (TRXs) (1–5). The most extensively studied enzymes regulated by this type of redox signaling mechanism reside in the chloroplasts of photosynthetic organisms, where the redox state of TRXs is indirectly influenced by light. Indeed, they are maintained in the reduced state by the photosynthetic electron transport through the action of photoreduced ferredoxin and ferredoxin-TRX reductase (6). In chloroplasts, TRXs are represented by several isoforms that are classified into five types: the f-, m-, x-, and y-types and the recently discovered z-type (7). Biochemical studies indicated that x- and y-type TRXs are mainly involved in the antioxidant response, whereas m- and f-type TRXs were proposed to specifically regulate enzymes involved in carbon metabolism (1, 2, 7, 8). By contrast, z-type TRX was reported to be specific for chloroplast transcription and development in land plants (9, 10). Among cellular processes regulated by TRX, the Calvin-Benson cycle (CBC), responsible for photosynthetic carbon fixation, contains 4 of 11 enzymes that are known to be regulated by dithiol/disulfide interchanges (2, 6, 7). These enzymes, namely glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoribulokinase, sedoheptulose 1,7-bisphosphatase, and fructose 1,6-bisphosphatase, are activated under

**Background:** Chloroplast PGK is a putative target of thioredoxin identified by redox proteomics.

**Results:** Chlamydomonas PGK1 is regulated by TRX-f via oxidoreduction of the Cys227−Cys361 disulfide bond.

**Conclusion:** Chlamydomonas PGK1 is a potentially new light-modulated Calvin-Benson cycle enzyme.

**Significance:** The complex redox regulation of the Calvin-Benson cycle and its evolution are further expanded.

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### Supplementary Data

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4. The abbreviations used are: TRX, thioredoxin; 3-PGA, 3-phosphoglycerate; Cbc, Calvin-Benson cycle; PGK, phosphoglycerate kinase; CrPGK1 and A4-GAPDH, respectively; DLS, dynamic light scattering; DTTrd and DTTox, reduced and oxidized DTT, respectively; DLS, dynamic light scattering; PDB, Protein Data Bank.
light and inactivated under dark conditions, when TRXs can function as reducing and oxidizing mediator, respectively. The regulation of these CBC enzymes is primarily controlled by f-type TRX (11–13).

In the last 2 decades, several proteomics-based approaches allowed the identification of a large number of putative TRX targets implicated in a myriad of cellular processes and pathways (3, 14, 15). These reports, coupled to biochemical studies, suggested that not only four but all enzymes involved in the CBC may withstand redox regulation through reversible disulfide bond formation or other cysteine-based redox modifications (2, 7, 16, 17). Among these enzymes, chloroplastic phosphoglycerate kinase (PGK) was identified by both TRX and glutaredoxin affinity columns (18–20). In addition, PGK from the unicellular green alga *Chlamydomonas reinhardtii* was recently reported to undergo glutathionylation, a redox modification involving a protein cysteine thiol and a molecule of glutathione (21, 22). These results strongly suggest that PGK might contain redox-responsive cysteine probably located on the molecular surface of the enzyme. Recently, two reports demonstrated that PGK from the diatom *Phaeodactylum tricornutum* and the cyanobacteria *Synechocystis* are inactivated by oxidation and that the oxidized enzymes are reactivated by TRX (23, 24). The redox sensitivity of both enzymes was proposed to involve oxidation to sulfenic acid of Cys77 in *P. tricornutum* PGK and the formation of disulfide bonds between Cys58 and Cys95 and between Cys314 and Cys340 in PGK from *C. reinhardtii* (CrPGK1) was recently reported to undergo glutathionylation, a redox modification involving a protein cysteine thiol and a molecule of glutathione (21, 22). These results strongly suggest that PGK might contain redox-responsive cysteines probably located on the molecular surface of the enzyme. Recently, two reports demonstrated that PGK from the diatom *Phaeodactylum tricornutum* and the cyanobacteria *Synechocystis* are inactivated by oxidation and that the oxidized enzymes are reactivated by TRX (23, 24).

In this work, we analyzed the effects of redox treatments on chloroplastic PGK from *C. reinhardtii* (CrPGK1), and we found that CrPGK1 activity is inhibited by the formation of a single regulatory disulfide bond. The activation of oxidized CrPGK1 was investigated by testing the efficiency of chloroplastic TRXs, and we established that CrPGK1 activation is specifically controlled by f-type TRX. Moreover, we determined that CrPGK1 oxidation affects the turnover number without altering substrate affinities. The identity of regulatory cysteines was investigated by site-directed mutagenesis, thiol titration, mass spectrometry analyses, and three-dimensional modeling. The results presented detail the molecular mechanism of the TRX-dependent redox regulation of CrPGK1 and propose the algal enzyme as a new CBC enzyme regulated by TRX.

**EXPERIMENTAL PROCEDURES**

**Material and Enzymes—**Modified porcine trypsin and recombinant Lys-C from *Pseudomonas aeruginosa* were obtained from Promega. NAP-5 columns and 5,5-dithiobis-2-nitrobenzoic acid were purchased from GE Healthcare and Pierce, respectively. All other chemicals were obtained from Sigma-Aldrich unless otherwise specified. Recombinant chloroplastic A₄-GAPDH from *C. reinhardtii* (CrGAPA) was expressed and purified as described previously (22).

**Expression and Purification of CrPGK1—**Recombinant CrPGK1 was produced using the pET vectors/BL21 expression system as described (22). Briefly, bacteria were grown in LB medium supplemented with 100 μg/ml ampicillin at 37 °C until the absorbance at 600 nm reached 0.5. The expression of CrPGK1 (wild-type protein and cysteine variants) was then induced by transfer to 27 °C for 18 h with the addition of 200 μM isopropyl–β-D-thiogalactopyranoside. The cells were harvested by centrifugation, resuspended in 30 mm Tris-HCl (pH 7.9), and broken using a French press (7×10⁷ Pa). Cell debris was removed by centrifugation, and the supernatant containing the soluble His-tagged proteins was loaded onto a Ni²⁺-HiTrap chelating resin (His-select nickel affinity gel; Sigma-Aldrich) pre-equilibrated with 30 mm Tris-HCl (pH 7.9) and 100 mm NaCl. The recombinant proteins were then purified according to the manufacturer’s instructions. The molecular mass and purity of CrPGK1 wild type (WT) was checked by SDS-PAGE after dialysis against 30 mm Tris-HCl (pH 7.9) and 1 mm EDTA. The concentrations of purified proteins were determined spectrophotometrically using a molar extinction coefficient at 280 nm of 20,065 M⁻¹ cm⁻¹. The resulting homogeneous proteins were stored at −20 °C.

**Site-directed Mutagenesis and Construction of CrPGK1-C108S, -C227S, and -C361S Mutant Forms—**Site-directed mutagenesis of Cys108, Cys227, and Cys361 to serine was performed using the QuikChange® site-directed mutagenesis kit (Stratagene) on the pET-3c-HIS/PGK vector obtained previously (22) and following the manufacturer’s instructions. The sequences of mutagenic complementary oligonucleotides were as follows: 5’-PGK C108S, ACCAAGGTCAATGCAGCATC- CCCGCCCGAGGTG; 3’-PGK C108S, CACCTCCGGGCCG-GTGCTTGCATCGACCCTTGGT; 5’-PGK C227S, GCGCTGATGGTGATGCTGCCCTTGA- CGGCCCCCGAGGTG; 3’-PGK C227S, GGCGTCAG. All mutations were confirmed by sequencing. The recombinant plasmids were introduced into Escherichia coli BL21 (DE3) for overexpression, and production and purification procedures were performed as described for the WT protein.

**Activity Measurements and Determination of Apparent Kinetic Parameters—**The catalytic activity of wild-type CrPGK1 and mutants was measured spectrophotometrically by a coupled...
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The enzyme assay with CrGAPA. The reaction mixture contained 30 mM Tris-HCl, pH 7.9, 10 mM KCl, 4 mM 3-PGA, 5 mM ATP, 20 μg/ml CrGAPA, and 0.15 mM NADH. The reaction was measured at 25 °C and initiated by the addition of CrPGK1. Activity was calculated from the decrease in absorbance at 340 nm (i.e., NADH oxidation). The apparent kinetic parameters (K_m and k_cat) for ATP and 3-PGA of reduced or oxidized wild-type CrPGK1 were determined at a fixed saturating concentration for one substrate and varying the concentration of the other substrate. Three independent experiments were performed at each substrate concentration, and the apparent K_m and k_cat values were calculated by nonlinear regression using the Michaelis-Menten equation with the program CoStat (CoHort Software).

Redox Treatments of Wild-type CrPGK1 and Cysteine Variants—Purified wild-type CrPGK1 and cysteine (CrPGK1-C108S, CrPGK1-C227S, and CrPGK1-C361S) were incubated at 25 °C for 30 min in the presence of 10 mM reduced DTT (DTTred) or 10 mM oxidized DTT (DTTox). After incubation, protein samples were assayed for PGK activity and separated by non-reducing SDS-PAGE (12% gel) and stained with Coomassie Brilliant Blue. The data were represented as the percentage of maximal PGK activity obtained by incubating the protein with DTTred.

Incubation of CrPGK1 with Chloroplastic TRXs from C. reinhardtii—Activation treatments of purified CrPGK1 WT was carried out incubating the enzyme in the presence of chloroplastic TRXs from C. reinhardtii supplemented with 0.2 mM DTTred. Control experiments were performed in the presence of 0.2 mM DTTred. At the indicated times, aliquots were withdrawn in order to assay PGK activity monitored as described above.

Kinetic Parameters of CrPGK1 Reduction in the Presence of f-type TRX—In order to establish the kinetic parameters of CrPGK1 reduction with f-type TRX, half-saturation concentrations (S_0.5 parameter, reflecting the concentration of f-type TRX needed to reach 50% enzyme activation) were determined by incubating (20 min) purified CrPGK1 with various concentrations of f-type TRX supplemented with 0.2 mM DTTred. The t_1/2 parameter (i.e., the time needed to reach 50% enzyme activation at the S_0.5 concentration) was determined by incubating purified CrPGK1 with f-type TRX at the S_0.5 concentration. At the indicated times, aliquots were withdrawn in order to assay PGK activity monitored as described above.

Determination of the Midpoint Redox Potential of CrPGK1—Redox titration experiments were carried out to monitor the redox state of the regulatory disulfide of CrPGK1 WT in the presence of mixtures of DTTred and DTTox to poise the redox potential. Recombinant CrPGK1 (5 μM) in its initial redox state was incubated for 2 h at 25 °C in 100 mM Tris-HCl, pH 7.9, 1 mM EDTA, 20 mM reduced/oxidized DTT in various dithiol/disulfide ratios and TRX-f (0.2 μM) in a final volume of 50 μl (29). Following incubation, PGK activity was assayed as described above. Results were fitted by non-linear regression (CoStat) to the Nernst equation (n = 2, one dithiol/disulfide (30)). Midpoint redox potentials are reported as means ± S.D. of two independent experiments.

Titration of Free Sulfhydryl (-SH) Groups—The number of free thiols in wild-type CrPGK1 (native or preactivated) and cysteine mutants (native) was determined spectrophotometrically under both non-denaturing and denaturing conditions with 5,5-dithiobis-2-nitrobenzoic acid (30). Briefly, 5–10 μM protein was added to a solution containing 0.2 mM 5,5-dithiobis-2-nitrobenzoic acid in 50 mM Tris-HCl (pH 7.9), 1 mM EDTA in the presence or absence of 5% SDS. After a 30-min incubation at 25 °C, the absorbance at 412 nm was determined. A molar extinction coefficient of 14,150 or 13,600 M⁻¹ cm⁻¹ was used to calculate the number of titratable sulfhydryl groups per monomer under non-denaturing or denaturing conditions, respectively.

MALDI-TOF Mass Spectrometry—Purified CrPGK1-WT and cysteine variants (15 μg) were alkylated with 10 mM N-ethylmaleimide and 10 mM iodoacetamide for 10 min in 20 μl of 50 mM ammonium bicarbonate. Then urea was added to reach 6 M, and alklylation was continued during 25 min. Urea was eliminated by ultrafiltration using Amicon Ultra centrifugal filter devices (molecular mass cut-off, 10 kDa), and CrPGK1 samples were further digested in 50 mM ammonium bicarbonate buffer for 4 h at 35 °C with a mixture of Lys-C and trypsin endoproteases (enzyme/substrate ratio = 1:37.5). After digestion, half of each sample was treated with 10 mM DTTred for 20 min. An aliquot of untreated and DTTred-treated CrPGK1 digests were mixed with half-saturated solution of α-cyano-4-hydroxycinnamic acid solution in 50% acetonitrile and 0.3% trifluoroacetic acid, and 1.5 μl of these mixtures were spotted on the MALDI plate. Acquisition of peptide mass fingerprints was achieved on a 4800+ MALDI-TOF/TOF mass spectrometer (ABSciex) after external calibration.

Dynamic Light Scattering (DLS)—DLS measurements were performed employing a Malvern Nano ZS instrument equipped with a 633-nm laser diode. Samples consisted of 0.5 ml of one DTTred-treated CrPGK1 WT (0.5 mg/ml) in 30 mM Tris-HCl (pH 7.9) and 1 mM EDTA. Protein samples were used in disposable polystyrene cuvettes of 1-cm optical path length, using water as a solvent. The width of DLS hydrodynamic diameter distribution is indicated by the polydispersion index. In the case of a monomodal distribution (Gaussian) calculated by means of cumulant analysis, polydispersion index = (σ/Z_{avg})², where σ is the width of the distribution, and Z_{avg} is the average diameter of the protein population, respectively.

Three-dimensional Modeling and Molecular Dynamics—The tridimensional model of the mature CrPGK1 protein (accession number A8JC04, 401 amino acids) was obtained using Swiss-Model software (31). The template was automatically searched with BLAST and HHblits, and the crystal structure of PGK from Bacillus steaerothermophilus (PDB code 1PH) was selected on the basis of sequence identity.

Using the coordinates of the tridimensional model of CrPGK1, its oxidized form was obtained via energy minimization by molecular mechanics using the AMBER software (32). In leap, the bond command was used to create a disulfide bond between Cys²²⁷ and Cys³⁶¹ (alchemical transformation of CYB227 and CYB361 residues in CYB227 and CYB361 residues in the AMBER force field (32)). The geometry optimization of the structure was then carried out using Sandor.
RESULTS

Sequence Analysis of Chlamydomonas PGK1—Multiple sequence alignments revealed that chloroplastic CrPGK1 exhibits relatively high similarity with PGKs from diverse plant and non-plant species (supplemental Fig. S1). CrPGK1 shows sequence identities in the range of 50–60% with human and bacterial PGKs, whereas the identity increases to 65–80% with PGKs from photosynthetic organisms (i.e. green algae, cyanobacteria, diatoms, and land plants) with the exception of PGK from the green alga Volvox carteri (92%, supplemental Fig. S1). CrPGK1 contains three cysteines, one of which (Cys108) is broadly conserved in all organisms except bacteria (Fig. 1). By contrast, Cys227 is widely conserved only in photosynthetic organisms, whereas Cys361 is conserved in some unicellular green algae (e.g. Chlorella variabilis) and also present in human PGK (absent in land plants, bacteria, diatoms, and Synechocystis sp. PCC 6803 PGK, F7URD6; PtPGK, P. tricornutum PGK, Q9M7P7; AtPGK, Arabidopsis thaliana PGK, P50318; SoPGK, Spinacia oleracea PGK, P29409; NtPGK, Nicotiana tabacum PGK, Q42961; ScPGK, Saccharomyces cerevisiae PGK, P70560; HsPGK, Homo sapiens PGK, P00558; BsPGK, B. stearothermophilus PGK, PDB code 1PHP. When conserved, cysteine residues are shown on a black background. Cysteine residues involved in the formation of the regulatory disulfide bonds in SyPGK and PtPGK are shown on a white background.

Effect of Reduced and Oxidized DTT on CrPGK1 Activity—In order to investigate the possible redox regulation of CrPGK1, the recombinant enzyme was expressed in E. coli and purified to homogeneity as described previously (22). The redox regulation of CrPGK1 was first explored by analyzing the effect of DTTred and DTTox on enzyme activity. As shown in Fig. 2A, incubation with DTTred strongly increased PGK activity compared with the untreated enzyme. By contrast, treatment with DTTox did not affect protein activity. These results suggest that the purified enzyme is already fully oxidized. The oxidized CrPGK1 retains 25% of its maximal activity and is fully activated by reducing treatments, leading to a 4-fold activation.

CrPGK1 Contains One or Several Intramolecular Disulfide Bond(s) Specifically Reduced by f-type TRX—To further investigate the redox state of CrPGK1, we analyzed the oxidized enzyme (i.e. untreated) by non-reducing SDS-PAGE. As shown in Fig. 2B, CrPGK1 was essentially monomeric (~45 kDa), indicating that no intermolecular disulfide bonds were present in the oxidized form of the enzyme. Subsequently, we examined the ability of TRX to catalyze CrPGK1 reduction using TRX-f from Chlamydomonas, a TRX specifically involved in the control of dithiol/disulfide exchange reactions of CBC enzymes (11–13). Control experiments were performed by incubating the purified enzyme in the presence of 0.2 mM DTTred, a concentration that does not allow CrPGK1 activation but maintains TRX in a reduced state (Fig. 3A). Under these conditions, CrPGK1 was rapidly activated by TRX-f and as efficiently as in the presence of 20 mM DTT. Moreover, activation kinetics were found to be dependent on TRX-f concentration (Fig. 3A). These results are consistent with the presence of one or several intramolecular disulfide bond(s) responsible for the TRX-dependent redox regulation of CrPGK1. The efficiency of TRX-f was further evaluated by measuring the concentration of TRX-f needed to allow 50% activation (S0.5) and the time needed to reach 50% activation at a TRX-f concentration corresponding to S0.5 (t0) (Fig. 4). The measured values, summarized in Table 1, were found to be similar to those previously determined for other TRX-f targets (34).

In addition to f-type TRX, the reduction of CrPGK1 was investigated in the presence of other types of TRX present in...
the chloroplast of *Chlamydomonas*, namely TRX-m, TRX-x, and TRX-y. As shown in Fig. 3B, none of these TRXs was able to efficiently activate CrPGK1, indicating that CrPGK1 is specifically regulated by f-type TRX, as observed previously for the other TRX-regulated CBC enzymes: GAPDH, phosphoribulokinase, fructose 1,6-bisphosphatase, and sedoheptulose 1,7-bisphosphatase (11–13).

**CrPGK1 Contains a Single Disulfide Bond with a Midpoint Redox Potential of $-335$ mV at the pH of Illuminated Chloroplasts**—The midpoint redox potential ($E_{m}$) of CrPGK1 was determined by measuring the activity of CrPGK1 equili-

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**TABLE 1**

| Kinetic parameters of f-type TRX-dependent activation of CrPGK1 |
|---------------------------------------------------------------|
| The values previously reported for activation by *Arabidopsis* TRX-f1 of sorghum NADP-malate dehydrogenase (NADP-MDH) and pea FBPase are provided for comparison (34). |
| $S_{0.5}$ | $t_{1/2}$ |
| CrPGK1 | 0.95 | 1 |
| NADP-MDH | 2.5 | 3.7 |
| FBPase | 0.4 | 0.9 |

<br>

**FIGURE 3.** Kinetics of activation of CrPGK1 in the presence of chloroplastic TRX from *C. reinhardtii* (CrTRX). A, activation of CrPGK1 in the presence of various concentration of f-type TRX. CrPGK1 was incubated in 30 mM Tris-HCl (pH 7.9) with 0.2 or 20 mM DTTred alone (closed circles or closed squares, respectively) or with 0.2 mM DTTred in the presence of 0.5 (open diamonds), 5 (open triangles), or 20 μM (open inverted triangles) f-type TRX. At the indicated times, aliquots were withdrawn in order to assay PGK activity. B, activation of CrPGK1 in the presence of chloroplastic *C. reinhardtii* TRXs. CrPGK1 was incubated in 30 mM Tris-HCl (pH 7.9) with 0.2 mM DTTred in the presence of different chloroplastic *C. reinhardtii* TRXs (5 μM) (gray bars). After a 20-min incubation, aliquots were withdrawn in order to assay PGK activity. Data are represented as mean percentage ± S.D. (error bars) of maximal PGK activity assayed after a 30-min incubation in the presence of 20 mM DTTred (n = 3). The activity of CrPGK1 oxidized (CrPGK1ox, white bar) and reduced (CrPGK1red, black bar) are also indicated.

**FIGURE 4.** Kinetic parameters of activation of CrPGK1 in the presence of f-type TRX. A, determination of half-saturating concentration ($S_{0.5}$) of f-type TRX. CrPGK1 was incubated in 30 mM Tris-HCl (pH 7.9) with 0.2 mM DTTred in the presence of various f-type TRX concentrations ranging from 0.5 to 20 μM. After 20-min incubations, aliquots were withdrawn in order to assay PGK activity. B, determination of half-time activation ($t_{1/2}$) of CrPGK1 in the presence of f-type TRX at $S_{0.5}$ concentration. CrPGK1 was incubated in 30 mM Tris-HCl (pH 7.9) with 0.2 mM DTTred in the presence of $-1$ μM TRX-f. At the indicated times, aliquots were withdrawn in order to assay PGK activity. Data are represented as mean percentage ± S.D. of maximal PGK activity assayed after a 30-min incubation in the presence of 20 mM DTTred (n = 3).

**TABLE 1**

| Kinetic parameters of f-type TRX-dependent activation of CrPGK1 |
|---------------------------------------------------------------|
| The values previously reported for activation by *Arabidopsis* TRX-f1 of sorghum NADP-malate dehydrogenase (NADP-MDH) and pea FBPase are provided for comparison (34). |
| $S_{0.5}$ | $t_{1/2}$ |
| CrPGK1 | 0.95 | 1 |
| NADP-MDH | 2.5 | 3.7 |
| FBPase | 0.4 | 0.9 |

**Disulfide Bond Formation on CrPGK1 Affects the Turnover Number**—In order to elucidate the effect of the redox state on CrPGK1 catalysis, the kinetic features of the enzyme were determined by comparing the activity of reduced (i.e. fully active) and oxidized (i.e. partially inhibited, ~25% residual activity) CrPGK1 in a coupled system with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The kinetic analysis was performed by using 3-PGA and ATP as variable substrates, and the kinetic parameters were calculated by non-linear regression.
analysis (Fig. 6). The results of these experiments revealed that CrPGK1 followed a Michaelis-Menten kinetic with an apparent $K_m$ for ATP ($\sim$0.36 mM) almost identical between the reduced and oxidized CrPGK1 (Fig. 6A and Table 2). Similarly, the apparent $K_m$ values for 3-PGA were highly comparable between the two forms (Fig. 6B and Table 2). These results indicate that the redox state of the protein does not influence its affinity for the two substrates. In addition, these values were comparable with those reported for PGK from other plant and non-plant sources (24, 39, 40). By contrast, the main difference between reduced and oxidized CrPGK1 relies on apparent turnover numbers ($k_{cat}$) that were found to be $\sim$84 and $\sim$30 s$^{-1}$, respectively (Table 2). This result strongly suggests that the formation of the disulfide bond affects protein activity in terms of product formation without altering the binding of both substrates. Consequently, the comparison of catalytic efficiencies ($k_{cat}/K_m$) between the two CrPGK1 forms revealed that the reduced enzyme had a 2.5-fold higher $k_{cat}/K_m$ compared with the oxidized enzyme (Table 2).

Analysis of CrPGK1 Redox Regulation by Site-directed Mutagenesis—In order to identify the cysteine residues involved in the redox regulation of chloroplastic CrPGK1 by dithiol/disulfide exchange, we used site-directed mutagenesis. CrPGK1 contains 3 cysteines, located at positions 108, 227, and 361, that were individually replaced by serines. The resulting recombinant cysteine variants CrPGK1-C108S, CrPGK1-C227S, and CrPGK1-C361S were purified to homogeneity and further analyzed for their sensitivity to redox treatments.

In contrast to the wild-type protein, the mutant lacking Cys$^{108}$ was found to be almost completely inactive under oxidizing conditions (DTT$^{ox}$) (Fig. 7). Nevertheless, a strong reactivation of CrPGK1-C108S was observed in the presence of DTT$^{red}$, suggesting that the absence of Cys$^{108}$ did not alter the sensitivity of the protein to the redox environment. However, the lack of basal protein activity observed for the purified enzyme probably suggests that Cys$^{108}$, although not involved in the formation of the regulatory disulfide bond, might have a structural role affecting the extent of protein inactivation upon disulfide bond formation. The analysis of CrPGK1-C227S and CrPGK1-C361S variants revealed that both proteins were constitutively active. Indeed, as shown in Fig. 7, the two mutant proteins were totally insensitive to oxidizing treatments, and no variation of protein activity was observed after incubation with DTT$^{red}$. Therefore, the absence of Cys$^{227}$ or Cys$^{361}$ allowed the respective mutant proteins to maintain a fully active conformation comparable with the reduced wild-type enzyme. Therefore, these results strongly suggest that Cys$^{227}$ and Cys$^{361}$ are the redox-responsive cysteines involved in the formation of the regulatory disulfide bond of CrPGK1.

Thiol Titration and MALDI-TOF Mass Spectrometry Confirm That the Regulatory Disulfide Bond Is Formed between Cys$^{227}$ and Cys$^{361}$—In order to confirm the presence of the Cys$^{227}$–Cys$^{361}$ disulfide bond, we carried out thiol titrations under both native and denaturing conditions using Ellman’s reagent (41) (Table 3). The results showed that reduced wild-type CrPGK1 contains two accessible cysteines in the native form, whereas three cysteines can be titrated after protein denaturation. By contrast, no accessible cysteines were found in the native oxidized enzyme, whereas one free thiol was detected under denaturing conditions. These results indicate that one cysteine is buried in the native conformation of both oxidized and reduced CrPGK1, and the two remaining cysteines are probably exposed to the solvent in the reduced protein but involved in a disulfide bond in the oxidized protein. Consistently, the two redox-insensitive mutants, CrPGK1-C227S and CrPGK1-C361S, contained one or two accessible cysteines under native or denaturing conditions, respectively, indicating that the buried cysteine is Cys$^{108}$. Accordingly, no accessible cysteines could be titrated for the oxidized CrPGK1-C108S variant either under native or denaturing conditions. All together these results are consistent with the formation of a regulatory disulfide bond between Cys$^{227}$ and Cys$^{361}$.

The identity of the cysteines involved in the formation of the regulatory disulfide bond was also investigated using MALDI-TOF peptide mass fingerprinting. Oxidized wild-type CrPGK1 and cysteine variant proteins were first alkylated with N-ethylmaleimide and iodoacetamide, incubated with proteases, and analyzed by mass spectrometry before and after incubation with DTT. Peptide mass fingerprinting of CrPGK1 revealed a peptide with a mass of 2507 Da corresponding to the sum of the Cys$^{227}$–Cys$^{361}$ regulatory disulfide bond. Indeed, it was not detected after incubation with DTT. Therefore, Cys$^{108}$–Cys$^{361}$ disulfide bond is most probably formed artifactual in the presence of the Cys$^{227}$–Cys$^{361}$ disulfide bond.

Figure 5. Redox titration of CrPGK1. The percentage of reduction was determined by measuring PGK activity after a 2-h incubation at 25 °C with 20 mM DTT in various dithiol/disulfide ratios. Results were fitted by non-linear regression to the Nernst equation for two electrons exchanged (n = 2) and one redox component. Data are represented as the mean percentage of triplicate determinations.


Redox Regulation of Chloroplastic PGK

![Graph](image_url)

**FIGURE 6. Kinetic analysis of CrPGK1.** A, variations of PGK activity (∆Abs₃₄₀/min) catalyzed by 20 nM CrPGK1 red (closed circles) or 20 nM CrPGK1 ox (open circles) at a fixed saturating concentration of 3-PGA (4 mM) and varying ATP concentrations. Data are represented as mean ± S.D. (n = 3). The kinetic parameters were calculated using nonlinear curve fit of the data sets. B, variations of PGK activity (∆Abs₃₄₀/min) catalyzed by 20 nM CrPGK1 red (closed circles) or 20 nM CrPGK1 ox (open circles) at a fixed saturating concentration of ATP (5 mM) and varying 3-PGA concentrations. Michaelis-Menten plots of PGK activity versus [3-PGA] is shown. Data are represented as mean ± S.D. (n = 3). The kinetic parameters were calculated using nonlinear curve fit of the data sets.

**TABLE 2**

| Protein         | 3-PGA | ATP     |
|-----------------|-------|---------|
|                 | Kₑₐₐ | kₑₐₐ   | kₑₐₐ/Kₑₐₐ |
| CrPGK1 red      | 0.4 ± 0.04 | 83.4 ± 0.5 | 2.09 × 10⁵ |
| CrPGK1 ox       | 0.31 ± 0.05 | 28.5 ± 0.5 | 0.92 × 10⁵ |

**FIGURE 7. Redox treatments of CrPGK1 cysteine mutants.** Effect of oxidized or reduced DTT on the activity of CrPGK1 WT and cysteine mutants. Purified recombinant proteins (CrPGK1 WT, C108S, C227S, and C361S) were incubated for 30 min in 30 mM Tris-HCl (pH 7.9) with 10 mM DTTox (white bars) or 10 mM DTTred (black bars) supplemented with 5 μM f-type TRX. After incubation, PGK activity was measured. Data are represented as mean percentage ± S.D. (error bars) of maximal activity of each cysteine variants assayed after 30 min incubation in the presence of 20 mM DTTred (n = 3).

The formation of this artifactual disulfide bond is probably due to partial alkylation of Cys¹⁰⁸. Indeed, the analysis of both CrPGK1-C361S and CrPGK1-C227S variants revealed that the derivatization of Cys¹⁰⁸ was not complete. Therefore, in the oxidized CrPGK1, the underderivatized Cys¹⁰⁸ probably attacks the Cys²²⁷–Cys³⁶¹ disulfide bond during denaturation and/or digestion of the protein and leads to the formation of the artifactual disulfide bond between Cys¹⁰⁸ and Cys³⁶¹. Taken together, these results indicate that CrPGK1 contains a single disulfide bond between Cys²²⁷ and Cys³⁶¹, which is responsible for the TRX-dependent redox regulation.

CrPGK1 is a Monomeric Enzyme with Two Distinct Domains—The biological conformation of CrPGK1 was evaluated by DLS measurements. The majority of structurally known PGKs are monomeric except PGK from *Pyrococcus horikoshii* (PDB code 3OZA (42)) and from *Pyrococcus horikoshii* OT3 (PDB code 2CUN)⁵ that are dimeric. The mean hydrodynamic radius measured for CrPGK1 was 3.39 ± 0.2 nm (mean ± S.D. of three independent experiments), corresponding to an apparent molecular mass of 58.8 kDa. This value is close to the molecular mass derived from the sequence (49.0 kDa), strongly suggesting that CrPGK1 is also a monomeric enzyme.

Because the atomic structure of CrPGK1 has not been determined, it was modeled on the basis of the crystal structure of *B. stearothermophilus* PGK (PDB code 1PHP (43)) sharing a sequence identity of 60% with CrPGK1. The obtained model corresponds to the reduced form of the enzyme because the reference structure has only one cysteine that does not display a conserved position in CrPGK1 (supplemental Fig. S1). The model building predicts a monomeric state for CrPGK1, in agreement with DLS results. Similarly to already known PGKs, the enzyme from *Chlamydomonas* is composed of two distinct domains, an N- and a C-terminal domain, both displaying a

⁵ H. Mizutani and N. Kunishima, unpublished results.
Rossmann fold and connected by a hinge region containing a long conserved α-helix (Fig. 9). The three cysteine residues, Cys108 belonging to the N-terminal domain and Cys227 and Cys361 both from the C-terminal domain, are all located in loop regions comprised either between a β-strand and an α-helix or between two α-helices (Fig. 9). The thiol group of Cys108 is oriented toward a hydrophobic cavity composed of phenylalanines (Phe20 and Phe135), leucines (Leu119 and Leu139), tyrosine (Tyr125), isoleucine (Ile100), and valine (Val122). Consistently, surface-accessible area calculations carried out using the coordinates of the CrPGK1 model showed that Cys108 is completely buried in the structure (surface-accessible area $1\text{Å}^2$), whereas Cys227 and Cys361 are both accessible, with surface-accessible area values of 9.9 and 16.3 $\text{Å}^2$, respectively. These findings fully support the thiol titration results obtained with Ellman’s reagent (Table 3). However, the three-dimensional model of reduced CrPGK1 showed that the thiol groups of the C-terminal cysteines (Cys227 and Cys361) are at a distance of about 10–15 $\text{Å}$, depending on the rotation of the side chains. Thus, the formation of a disulfide bond between these residues necessarily entails a conformational rearrangement of the enzyme made feasible by their positioning in non-structured portions.

**TABLE 3**
Number of free thiols in CrPGK1 WT and cysteine variants under both native and denatured conditions

| Conditions | WT | WTred | C108S | C227S | C361S |
|------------|----|-------|-------|-------|-------|
| Native     | 0.25 ± 0.08 | 1.94 ± 0.12 | 0.12 ± 0.02 | 1.00 ± 0.1 | 0.81 ± 0.09 |
| Denaturing | 1.29 ± 0.15 | 2.96 ± 0.12 | 0.22 ± 0.02 | 2.13 ± 0.1 | 1.88 ± 0.03 |

**DISCUSSION**

In photosynthetic organisms, proteomic approaches previously identified chloroplastic PGK among putative TRX targets (18, 19). This suggested that PGK might undergo, as established for four other CBC enzymes, light-dependent modulation of enzyme activity via dithiol/disulfide interchanges under the control of TRX. The aim of the present study was to characterize the redox features of chloroplastic PGK from *Chlamydomonas* and to investigate whether the enzyme contains redox-sensitive cysteines.

Biochemical analyses revealed that purified CrPGK1 has a low activity and is activated after treatment with DTTred, indicating that the enzyme has a basal activity in the oxidized form (25% of the maximal activity) and becomes fully active after reduction. A complete and fast activation of CrPGK1 was achieved in the presence of reduced f-type TRX. Redox titrations revealed that CrPGK1 contains a single regulatory disulfide bond with a low midpoint redox potential ($-335 \text{mV at pH 7.9}$). It is therefore concluded that the level of activity of CrPGK1 is dependent on the redox state of two cysteine residues that can form a disulfide bond and that CrPGK1 might constitute an additional light-modulated CBC enzyme with a
low activity in the dark and a TRX-dependent activation in the light.

It has been previously demonstrated that the regulation of the four CBC enzymes undergoing dithiol/disulfide interchanges is preferentially controlled by f-type TRX, which is considered the specific redox mediator of the photosynthetic carbon fixation pathway (2, 6, 7). Redox activation of CrPGK1 was also found to be specifically controlled by f-type TRX because other chloroplastic TRX types proved inefficient. The kinetic parameters determined for CrPGK1 reduction were very similar to those previously determined for other TRX-f targets. No consensus sequence around the regulatory disulfide bond can be distinguished among TRX-f-dependent enzymes, but in the case of fructose 1,6-bisphosphatase, the strict specificity for f-type TRX was proposed to depend on surface protein charges that would allow specific protein-protein interactions between fructose 1,6-bisphosphatase and TRX-f (44). However, because the distribution of charges at the protein surface does not seem to be conserved among TRX-f targets, it is not possible to determine whether these charges contribute to the TRX specificity in the case of CrPGK1 or if other structural determinants are involved.

The analysis of the three-dimensional model of CrPGK1 suggested that the three cysteine residues have different surface accessibility, and only Cys\textsuperscript{227} and Cys\textsuperscript{361} are solvent-exposed. Although these two residues are not close enough to spontaneously form a disulfide bond, their distance is compatible with a TRX-induced oxidation that could drive disulfide bond formation. In order to identify the cysteines involved in the redox regulation of CrPGK1, we generated three mutant proteins in which each cysteine was replaced by serine (CrPGK1-C108S, CrPGK1-C227S, and CrPGK1-C361S). Using a combination of activity assays, thiol titrations, and mass spectrometry analyses of these cysteine variants, we demonstrated that Cys\textsuperscript{227} and Cys\textsuperscript{361} are involved in the formation of the regulatory disulfide bond.

Kinetic analyses clearly showed that the redox state of the enzyme does not affect the binding affinity of CrPGK1 toward either the cofactor ATP or the substrate 3-PGA (Table 2). By contrast, the turnover number is strongly diminished for the oxidized enzyme, and its catalytic efficiency is decreased by 2.5-fold compared with the reduced form. These experimental findings suggest that conformational changes linked to formation of the Cys\textsuperscript{227}—Cys\textsuperscript{361} disulfide might be responsible for partial inhibition upon protein oxidation. Similarly, it has been shown that photosynthetic GAPDH (heterotetrameric isozyme) underwent TRX-dependent disulfide bond formation without modification of the Michaelis-Menten constants for either the cofactor (NADPH) or the substrate (1,3-bisphosphoglycerate) (45–47).

In order to shed light on the possible structural changes occurring upon CrPGK1 oxidation, we carried out molecular mechanic calculations. This analysis was performed using the model of the reduced protein and imposing the formation of the disulfide bond between Cys\textsuperscript{227} and Cys\textsuperscript{361} by energy minimization to adjust the protein conformation. It has been demonstrated for human PGK that after binding of the two substrates, the enzyme assumes a half-closed conformation in which the two substrates appear too distant from each other to allow phosphoryl transfer (28). A significant “hinge bending” movement, which leads the enzyme to a closed conformation, was suggested to allow enzyme catalysis to occur. Molecular mechanic calculations show that when the two regulatory cysteines of CrPGK1 approach to form a disulfide bond, a confor-
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**FIGURE 10.** Structural superimposition of the reduced and oxidized models of CrPGK1 to the structure of human PGK1 in the fully closed conformation. The reduced (salmon) and the oxidized (cyan) models of CrPGK1 are superimposed onto the crystal structure of the fully closed human PGK1 in complex with 3-PGA, MgF$_3^-$, and ADP (gray, PDB code 2WZB). The portion of the central $\beta$-sheet of the C-terminal domain is shown. The formation of the disulfide bridge in CrPGK1 introduces a constraint in the enzyme structure, causing a movement of strands $\beta$9 and $\beta$10 in the opposite direction with respect to the rotation necessary for enzyme closure and catalysis.

The redox sensitivity of chloroplastic PGK from *Chlamydomonas*, representing the fifth enzyme known to be redox-regulated by TRX-dependent mechanisms, constitutes another step toward the understanding of the regulation of the CBC. Similarly, PGKs from *Synechocystis* and *P. tricornutum* were found to be inactivated under oxidizing conditions through the formation of one or several disulfide bond(s) (23, 24). However, whereas the *Chlamydomonas* enzyme resembles classical TRX-regulated CBC enzymes from land plants activated under illumination, the *Synechocystis* enzyme is only inactivated in the presence of strong oxidants, suggesting that the CBC may be regulated by light in *Chlamydomonas* and by oxidants in cyanobacteria. A similar difference was observed for redox regulation of cyanobacterial phosphoribulokinase (49). Nevertheless, the structural determinants of the regulation are different for each PGK because the regulatory cysteines are not conserved (Fig. 1). This is particularly clear with diatom PGK, where the two regulatory cysteines are located in the N-terminal domain of the protein (Cys$_{58}$ and Cys$_{95}$). By contrast, the two redox-sensitive cysteines (Cys$_{114}$ and Cys$_{340}$) of *Synechocystis* PGK, although not conserved in the primary sequence (Fig. 1), are located in the C-terminal domain of the enzyme as in *Chlamydomonas* PGK. To date, it is not known whether land plant PGK is also subjected to a redox control involving dithiol/disulfide interchanges as observed for PGK from lower photosynthetic organisms. The comparison of CrPGK1 with homologous enzymes from *Arabidopsis* and spinach revealed that land plant PGKs only contain Cys$_{108}$ and Cys$_{227}$, whereas the redox-sensitive Cys$_{361}$ is absent, suggesting that the redox regulation of chloroplastic PGK might not be conserved in land plants. Alternatively, the two cysteinyl residues of land plant PGKs might be implicated in the formation of intermolecular disulfide bonds, allowing protein regulation through redox-dependent monomer-oligomer interconversion. Further studies are therefore required to investigate the redox regulation of PGK from land plants and to evaluate the underlying molecular mechanisms.

Chloroplastic PGK participates, together with photosynthetic GAPDH, in the reductive step of the Calvin-Benson cycle, where 3-PGA is reduced to glyceraldehyde 3-phosphate using ATP and NADPH. In *Chlamydomonas*, photosynthetic GAPDH is a homotetrameric enzyme (A$_4$-GAPDH) composed by A-type subunits, and its activity is regulated through the light-dependent formation of a supramolecular complex with the small redox peptide CP12 and phosphoribulokinase (50, 51). A similar regulation also occurs for land plant GAPDH, but this mechanism only concerns the homotetrameric isoform that represents only 20% of total photosynthetic GAPDH (52). The remaining 80% is constituted by a heterotetrameric enzyme (AB-GAPDH) composed of A- and B-type subunits, the latter being responsible for an autonomous redox regulation involving the formation of a TRX-dependent disulfide bond (29, 45). In addition, AB-GAPDH is also regulated via interconversion between active heterotetramers and low activity oligomers (29, 47), and this modification of quaternary structure is controlled by different protein ligands, including NAD(P)(H), ATP, and 1,3-bisphosphoglycerate. It is therefore clear that the redox regulation of photosynthetic GAPDH became more sophisticated after land colonization. Based on these considerations, we cannot exclude the possibility that also
land plant PGK might be regulated through similar or more complex redox mechanisms compared with those determined for the algal enzyme. However, it is also possible that evolution between algae and land plants favored the implementation of GAPDH regulatory mechanisms despite a loss of PGK regulation. This may also be true for some algae because Cys361 is also absent in the pluricellular alga *V. carteri*. This second hypothesis is particularly intriguing because redox regulation has been considered to increase in complexity during evolution, and it would constitute the first example of a CBC enzyme that would be redox-regulated in lower photosynthetic organisms but not in terrestrial plants.

Besides being regulated by TRX-dependent dithiol/disulfide interchange, chloroplastic PGK from *C. reinhardtii* was recently identified as a potential target of other redox post-translational modifications such as S-thiolation, glutathionylation, and nitrosylation (17, 21, 22). Both Cys108 and Cys361 were found to undergo glutathionylation, suggesting that Cys108 may also play a regulatory role under specific conditions or in the presence of additional proteins (22). Two recent reports also identified PGK from land plants as a putative target of nitrosylation (53, 54). These additional redox post-translational modifications started to be recognized as important regulatory mechanisms involved in the control of numerous cellular plant processes under stress conditions (16, 55–58). Further studies will therefore be required to determine whether chloroplastic PGK is tightly controlled by multiple redox-regulatory mechanisms, to analyze how the complex interplay between varying environmental conditions and the intracellular redox state determine the type and the extent of each redox modification and to understand how these mechanisms contribute to the fine tuning of carbon fixation in photosynthetic organisms.

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