Biochemical Characterization of Glutaredoxins from *Chlamydomonas reinhardtii* Reveals the Unique Properties of a Chloroplastic CGFS-type Glutaredoxin*

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Glutaredoxins (GRXs) are small ubiquitous disulfide oxidoreductases known to use GSH as electron donor. In photosynthetic organisms, little is known about the biochemical properties of GRXs despite the existence of ∼30 different isoforms in higher plants. We report here the biochemical characterization of *Chlamydomonas* GRX1 and GRX3, the major cytosolic and chloroplastic isoforms, respectively. Glutaredoxins are classified on the basis of the amino acid sequence of the active site. GRX1 is a typical CPYC-type GRX, which is reduced by GSH and exhibits disulfide reductase, dehydroascorbate reductase, and deglutathionylation activities. In contrast, GRX3 exhibits unique properties. This chloroplastic CGFS-type GRX is not reduced by GSH and has an atypically low redox potential (-323 ± 4 mV at pH 7.9). Remarkably, GRX3 can be reduced in the light by photoreduced ferredoxin and ferredoxin-thioredoxin reductase. Both GRXs proved to be very efficient catalysts of A_4-glyceraldehyde-3-phosphate dehydrogenase deglutathionylation, whereas cytosolic and chloroplastic thioredoxins were inefficient. Glutathionylated A_4-glyceraldehyde-3-phosphate dehydrogenase is the first physiological substrate identified for a CGFS-type GRX.

GSH is the main low molecular weight thiol in cells and is generally considered to constitute a redox buffer. Besides its antioxidant functions, GSH is also important for the detoxification of xenobiotics and heavy metals (1). Moreover, glutathione has recently been found to be involved in a post-translational modification termed glutathionylation (2, 3). This modification, which occurs under oxidative stress conditions, consists of the reversible formation of a mixed disulfide between glutathione and a cysteine residue on the target proteins. Glutathionylation can alter, either positively or negatively, the activity of several proteins in mammals. Compared with mammals, bacteria, and yeast, very little is known about glutathionylation in photosynthetic organisms. However, recent studies have allowed the identification of a growing number of plant proteins undergoing glutathionylation (4–11). The precise mechanisms leading to the formation of protein glutathione mixed-disulfides *in vivo* are largely unknown. On the contrary, the reverse reaction, named deglutathionylation, is likely catalyzed by glutaredoxins (GRXs)[2] (12).

GRXs are small ubiquitous disulfide oxidoreductases that belong to the thioredoxin (TRX) superfamily and generally bear a Cys-*X*-*X*-Cys/Ser active site. Using reduced glutathione as a reductant, they can catalyze thiol-disulfide redox reactions using two distinct mechanisms. The reduction of protein disulfides occurs through a dithiol mechanism that requires both active site cysteines (13). On the other hand, the reduction of glutathione-mixed disulfides can occur either through a monothiol mechanism, which only requires the N-terminal active site cysteine residue, or through a dithiol mechanism involving an additional cysteine (13, 14). GRXs also possess a dehydroascorbate reductase (DHAR) activity (15, 16). Classic GRXs have a redox potential between -190 and -230 mV at pH 7 (17). Deglutathionylation was shown to be catalyzed more efficiently by GRX than by other disulfide oxidoreductases such as TRX or protein disulfide isomerase from human or *Escherichia coli* (18, 19). However, considering the multiplicity of TRX isoforms in photosynthetic organisms (20), it would be interesting to test whether one of these TRX might be able to catalyze deglutathionylation.

GRXs are encoded by a multigenic family comprising ∼30 members in higher plants (21, 22). The number is more limited in lower photosynthetic eukaryotes such as the unicellular green alga *Chlamydomonas reinhardtii*, which contains six GRXs (21). Plant GRXs have been divided into different types called CPYC, CGFS, and CC. The CC type, which comprises ∼20 members, is specific to higher plants. The CPYC type corresponds to "classic" GRXs possessing disulfide oxidoreductase, DHAR, and deglutathionylation activities. Cytosolic poplar GRXs, the only plant GRXs characterized to date, are all CPYC-type, have DHAR and disulfide reductase activities, and are able to reduce type II peroxiredoxins (23–25). Moreover, one of these GRXs was recently shown to bind an iron-sulfur cluster (26, 27). *Chlamydomonas* contains two members of

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[2] The abbreviations used are: GRX, glutaredoxin; TRX, thioredoxin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DHA, dehydroascorbate; BPGA, 1,3-bisphosphoglycerate; FTR, ferredoxin-thioredoxin reductase; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; HED, 2-hydroxyethyldisulfide; DHAR, dehydroascorbate reductase.

this family, GRX1 and GRX2. No GRX with a classic CPYC active site is predicted to be present in chloroplasts (21, 22), whereas a number of chloroplastic proteins, including TRXf (9) and A_4-GAPDH (11) have been shown to undergo glutathionylation.

Chloroplasts contain GRXs belonging to the CGFS group, four members of this group being present in both *Arabidopsis* and *Chlamydomonas*. This type of GRX is ubiquitous and corresponds to *E. coli* GRX4 and yeast GRX3, -4, and -5. In yeast, GRX5 is a mitochondrial GRX required for the assembly/biogenesis of iron-sulfur clusters (28), whereas GRX3/4 are nuclear targeted and were shown to regulate the nuclear localization of the transcription factor Aft1 (29, 30). Biochemical data on CGFS-type GRXs are limited to studies on yeast GRX5 (14) and *E. coli* GRX4 (31), which revealed the presence of a disulfide bond between the cysteine of the CGFS active site and a second cysteine located in the C-terminal part of these proteins. The redox potential of yeast GRX5 was measured to be −175 mV (14). Yeast GRX5 was found to be able to catalyze protein-glutathione mixed disulfide reduction, but the physiological reductant of yeast GRX5 remains to be identified, whereas *E. coli* CGFS GRX4 was recently shown to be reduced by NADPH-thioredoxin reductase (31). *Chlamydomonas* contains CGFS-type GRXs homologous to yeast GRX5 and yeast GRX3/4, named GRX5 and GRX4, which are predicted to be located in the mitochondria and the cytosol, respectively. The two other CGFS-type GRXs, GRX3 and GRX6, are predicted to be targeted to chloroplasts. GRX3 is a small GRX, similar to yeast GRX5, whereas GRX6 contains a C-terminal GRX domain and an N-terminal domain of unknown function. The plastidial localization of GRX3 homologue in *Arabidopsis*, GRX-S14, was recently confirmed experimentally (32).

Here we report the biochemical characterization of *Chlamydomonas reinhardtii* GRX1 and GRX3. GRX1 is the major cytosolic GRX and belongs to the classic CPYC type, whereas GRX3 is a chloroplastic protein, homologous to higher plants GRX-S14 and belonging to the CGFS type. GRX3 is the most highly expressed GRX in *Chlamydomonas* and likely represents the major chloroplastic GRX (21). For both GRXs, the kinetic parameters of the DHAR and disulfide reductase activities were measured. Deglutathionylation assays were performed with the standard HED assay but also using glutathionylated A_4-GAPDH as a substrate. The efficiency of both GRX in the A_4-GAPDH deglutathionylation assay were determined and compared with those of different types of plant TRXs. The results show that GRX1 possesses biochemical properties similar to CPYC-type GRXs from other organisms but that GRX3 exhibits unique properties, notably, the ability to be very efficiently reduced in the light by ferredoxin-thioredoxin reductase.

EXPERIMENTAL PROCEDURES

Materials—NAP-5 columns were purchased from GE Healthcare. HED and DTNB were from Aldrich and Pierce, respectively. All the other reagents were from Sigma.

Plasmid Construction for Expression of GRX1 and GRX3 in E. coli—*C. reinhardtii* GRX1 and GRX3 correspond to expressed sequence tag sequences AV623478 and AV390622,

respectively. The corresponding cDNA clones were obtained from the Kazusa DNA Research Institute (Chiba, Japan) and used to amplify the sequence encoding the mature form of GRX1 and GRX3 by PCR. Specific endonuclease sites (bold) were introduced at the 5′- and 3′-ends of the coding sequences using the following primers: 5′NdeI-GAACAAC-**CATATG**CTCGCTACTCGTTCTGCTGC and 3′BamHI-GGTC**GGATCC**TTACAGAGCACCGGCCTCCT for CrGRX1; 5′NcoI-CGCG**CCATGG**CTTCTGGAATGGCCCCCGA and 3′BamHI-TTCT**GGATCC**CTAGGAGTTGAGGGCAATCT for CrGRX3.

The sequences encoding CrGRX1 and the putative mature form of CrGRX3 were cloned in a modified pET-3c or pET-3d vector containing additional codons upstream of the NdeI/NcoI site so as to express the protein with a polyhistidine tag at the N terminus. The sequence was checked by sequencing.

Protein Expression and Purification—The pET vectors allowing expression of recombinant GRX1 and GRX3 were transformed in *E. coli* BL21(DE3) cells, which were grown in LB medium supplemented with 100 μg/ml ampicillin at 37 °C. Protein expression was induced at an A_{600} of 0.5 with 0.2 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37 °C or for 18 h at 30 °C for CrGRX1 and CrGRX3, respectively. Cells were harvested by centrifugation at 3,000 × g for 15 min, resuspended in 30 mM Tris-HCl, pH 7.9, broken by three passages through a French press (6.9 × 10^7 Pa), and cleared by centrifugation at 39,000 × g for 30 min. The supernatants were applied onto a Ni^{2+} HiTrap chelating resin (HIS-Select® Nickel Affinity Gel, Sigma) pre-equilibrated with 30 mM Tris-HCl, pH 7.9. The recombinant proteins were then purified according to the manufacturer's instructions. The molecular mass and purity of the proteins were analyzed by SDS-PAGE after dialysis against 30 mM Tris-HCl, pH 7.9, 1 mM EDTA. The protein concentrations of GRX1 and GRX3 were determined spectrophotometrically using molar extinction coefficients at 280 nm of 4,595 $M^{-1}cm^{-1}$ and 11,585 $M^{-1}cm^{-1}$, respectively. The resulting homogeneous proteins were stored at −20 °C. *A. thaliana* NTRC coding sequence was cloned in pET-16b, and the protein was expressed and purified as described above for GRX3. The other recombinant proteins were produced and purified as previously described for *C. reinhardtii* TRXh1 (33), *C. reinhardtii* ferredoxin (34), *A. thaliana* NADPH-thioredoxin reductase (35), *A. thaliana* A_4-GAPDH (36), and *Synechocystis sp.* FTR (37). Thylakoids from *Chlamydomonas* were isolated as described in a previous study (9).

Dehydroascorbate Reductase Activity—The DHAR activity of GRX was carried out using a modified version of the standard thioltransferase activity (HED assay). The reaction contained 0.1 M potassium phosphate buffer, pH 7.0, 2 mM EDTA, 2 mM GSH, 0.1 mg/ml bovine serum albumin, 6 μg/ml yeast glutathione reductase, 200 μM NADPH, and 1 mM dehydroascorbate (DHA). After 1 min of preincubation, varying concentrations of GRX were added to the sample cuvette, while buffer was added to the reference cuvette. The decrease in absorbance at 340 nm was followed spectrophotometrically. GRX activity was determined after subtracting the spontaneous reduction of DHA by GSH observed in the absence of glutaredoxin. Activity was expressed as micromoles of NADPH oxidized/min. The appar-

ent K_m value for dehydroascorbate was determined using a DHA concentration range from 0.125 to 1 mM in the presence of 2 mM GSH. Three independent experiments were performed at each substrate concentration, and the apparent K_m and k_{cat} values were calculated by non-linear regression using the program CoStat (CoHort Software).

Turbidimetric Assay of Insulin Disulfide Reduction—The rate of insulin disulfide reduction by *Chlamydomonas* TRXh1, GRX1, and GRX3 was monitored spectrophotometrically following the turbidity at 650 nm. The reaction mixture contained 0.1 M potassium phosphate buffer, pH 7.0 or 7.9, 0.13 mM bovine insulin (0.75 mg/ml), 2 mM EDTA, and 5 μM of each redoxin (TRXh1, GRX1, or GRX3). The reaction was started by adding 0.33 mM DTT or 1 mM GSH as reductants. The non-enzymatic reduction of insulin by DTT or GSH was used as a control.

Determination of Glutaredoxin Activity (HED Assay)—A mixture of 1 mM GSH, 0.2 mM NADPH, 2 mM EDTA, 0.1 mg/ml bovine serum albumin, and 6 μg/ml yeast glutathione reductase was prepared in 100 mM Tris-HCl, pH 7.9. To 500 μl of this mixture, HED was added at a final concentration of 0.7 mM. After 3 min glutaredoxin was added to the sample cuvette, and an equal amount of buffer to the reference cuvette. The decrease in absorbance at 340 nm was followed using a HITACHI U-3000 spectrophotometer. GRX activity was determined after subtracting the spontaneous reduction rate observed in the absence of glutaredoxin. Activity was expressed as micromoles of NADPH oxidized/min. The K_m value for glutathione was determined using varying concentrations of GSH (0.5–3.5 mM) and 10 nM GRX1 in the presence of 0.7 mM HED. Three independent experiments were performed at each substrate concentration, and the apparent K_m and k_{cat} values were calculated by non-linear regression using the program CoStat.

Analysis of Protein Thiol Content in C. reinhardtii GRX3—GRX3 samples (50 μM) were reduced or oxidized with 20 mM reduced DTT or oxidized DTT for 1 h at 25 °C followed by desalting on NAP-5 columns (Sephadex G-25) equilibrated with 30 mM Tris-HCl, pH 7.9. Oxidized GRX3 was subsequently treated with 5 mM GSH for 1 h at 25 °C, followed by desalting on NAP-5 columns. After desalting, the number of free thiols in GRX3 protein was determined spectrophotometrically with DTNB (38). Briefly, 20–30 μM of protein were added to a solution containing 200 μM DTNB in 30 mM Tris-HCl, pH 7.9. After 10 min at room temperature the absorbance at 412 nm was determined. A molar extinction coefficient of 14150 M^{-1}cm^{-1} was used to calculate the number of titrated sulfhydryl groups.

Determination of the Redox Potential of GRX3—Before each redox titration experiment, GRX3 was desalted in 100 mM Tricine-NaOH, pH 7.9, using NAP-5 columns. Redox titration experiments were performed with 60 μM GRX3 incubated for 3 h at 25 °C with 20 mM DTT at various dithiol-disulfide ratios, in a total volume of 500 μl. Following incubation, samples were desalted using NAP-5 columns equilibrated with 100 mM Tricine-NaOH, pH 7.9. To avoid any possible DTT contamination, only 700 μl of eluted samples (1 ml) was collected. Absorbance at 280 nm was recorded immediately after desalting, subsequently 0.2 mM DTNB was added and the absorbance at 412

nm was measured. Control experiments were performed under the same conditions but in the absence of GRX3. The number of free thiol groups under different redox conditions was calculated from the ratio between the absorbance at 412 nm (molar extinction coefficient: 14150 M^{-1}cm^{-1}) and the absorbance at 280 nm (molar extinction coefficient: 11585 M^{-1}cm^{-1} for CrGrx3). The titration results were fitted by non-linear regression (CoStat) to the Nernst equation setting the value of n to 2 (the disulfide-dithiol is expected to be a two-electron transfer process), according to previous studies (39, 40). The redox potential is reported as mean ± S.D. of triplicate experiments.

Deglutathionylation Assays—A$_4$-GAPDH was glutathionylated as described before (11). Briefly, the protein (2.5 μM) was incubated with 0.1 mM H$_2$O$_2$ in the presence of 0.5 mM GSH in 100 mM Tricine-NaOH, pH 7.9. After 15-min incubation, aliquots were withdrawn and 1,3-bisphosphoglycerate (BPGA, produced by mixing 5 units/ml of 3-phosphoglycerate kinase, 2 mM ATP, and 3 mM 3-phosphoglycerate) was added to prevent enzyme activity from any further inactivation. Deglutathionylation assays were performed in the following conditions: I, 20 mM reduced DTT and 5 mM GSH as control; II, NADPH-thioredoxin system (0.3 mM NADPH, 0.2 μM *A. thaliana* NADPH-thioredoxin reductase B plus varying concentrations of TRXh1); III, GSH-glutaredoxin system (5 mM GSH plus 5 μM GRX3 or variable amounts of GRX1); and IV, variable concentrations of GRX3 in the presence of the complete reconstituted light activation system comprising thylakoids, ferredoxin, and ferredoxin-thioredoxin reductase as described (9). At indicated times aliquots were withdrawn to assay enzyme activity and monitored as described previously (11).

RESULTS

GRX3 Contains an Intramolecular Disulfide—Yeast GRX5 and *E. coli* GRX4 are CGFS-type GRXs, which have been shown to contain a disulfide between the cysteine of the CGFS active site and a cysteine located in the C-terminal part of the protein (14, 31). This C-terminal cysteine is conserved in many CGFS-type GRX, including *Chlamydomonas* GRX3 (21). To determine if GRX3 contains a disulfide, the purified enzyme was incubated with reduced or oxidized DTT, and the number of free thiols was quantified using DTNB. Although reduced GRX3, whose amino acid sequence includes two Cys, was experimentally found to contain two reactive thiols (1.98 ± 0.18), GRX3 treated with oxidized DTT contained 0.34 ± 0.05 free thiols. Moreover, oxidized and reduced GRX3 appear as monomers on SDS-PAGE performed in reducing and non-reducing conditions (data not shown). Taken together, these results indicate that GRX3 contains an intramolecular disulfide bond between the two cysteines of the protein and therefore may be able to catalyze disulfide bonds reduction through either a monothiol or a dithiol mechanism.

DHAR Activity—Because GRXs have the ability to catalyze the reduction of DHA by glutathione (15, 16), we tested whether *Chlamydomonas* GRX1 and GRX3 were able to accelerate the spontaneous reduction of dehydroascorbate by GSH. To minimize the rapid non-enzymatic reduction of DHA by GSH at high pH values, the assays were performed at pH 7.0. The reaction was followed by measuring the NADPH oxidation
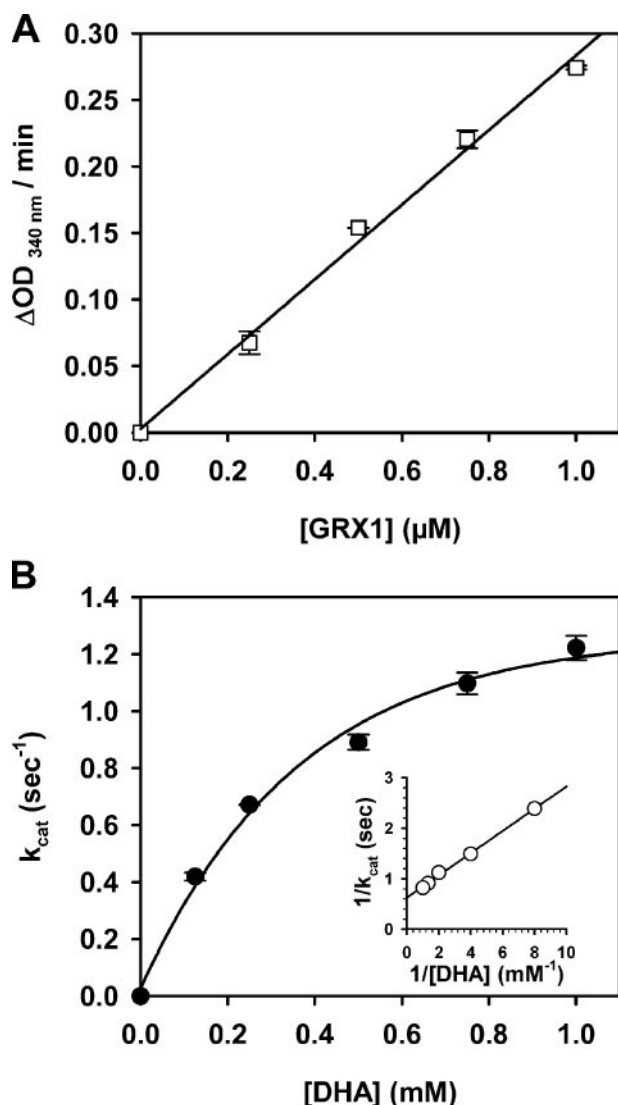
FIGURE 1. **DHAR activity of *Chlamydomonas* GRX1.** *A*, linear dependence of DHAR activity on GRX1 concentration expressed as A_{340}/min. The data are represented as mean $\pm$ S.D. *B*, variation of apparent turnover number (s^{-1}) during DHAR activity catalyzed by 0.5 μM GRX1 in the presence of varying DHA concentrations. Turnover represents moles of NADPH oxidized/s in the presence of 1 mol of GRX1. The data are represented as mean $\pm$ S.D. The best fit was obtained using the Michaelis-Menten equation.

TABLE 1

Kinetic parameters of *Chlamydomonas* GRX1 in the DHA reductase assay

The apparent K_m value was determined using a DHA concentration range of 0.125–1 mM in the presence of 2 mM GSH. The apparent K_m and apparent turnover values (k_{cat}) were calculated by non-linear regression using the Michaelis-Menten equation. For details see "Experimental Procedures." Data are represented as mean $\pm$ S.D.

|	K_m	k_{cat}	k_{cat}/K_m
	mM	s^{-1}	$M^{-1}s^{-1}$
GRX1	0.39 ± 0.03	1.67 ± 0.09	4.3×10^3

in a coupled system as described under "Experimental Procedures." First, we analyzed the dependence of DHAR activity on glutaredoxin concentrations. The DHAR activity of GRX1 displayed a linear relationship with increasing protein concentrations in the 0–1 μM range (Fig. 1). The kinetic analysis of the DHAR activity revealed an apparent K_m value of 0.39 ± 0.03

mM for DHA and a k_{cat} of 1.67 ± 0.09 s^{-1} (Table 1). These values are comparable to those reported for other CPYC-type GRXs (15, 16). In contrast to GRX1, GRX3 was totally unable to reduce DHA into ascorbic acid (data not shown) as previously described for yeast GRX5 (14).

Insulin Reduction by TRXh1, GRX1, and GRX3—Insulin reduction is a classic assay to test disulfide reductase activity (41). This activity has been used to determine whether the two GRXs were able to reduce disulfide-bridged insulin, using cytosolic TRXh1 from *Chlamydomonas* as a control. As expected, TRXh1 started to efficiently reduce insulin after a lag phase of ∼6–8 min (Fig. 2*A*). Although much less efficient than TRXh1, GRX1 was able to reduce insulin disulfides in the presence of DTT (Fig. 2*B*). In addition, GRX1 also reduced insulin disulfides using GSH as a reductant but much less efficiently than in the presence of DTT. By contrast, GRX3 did not display any activity in the insulin assay (Fig. 2*C*). Indeed, incubation of insulin and GRX3 in the presence of DTT as an electron donor, at either pH 7.0 or pH 7.9, did not reveal any significant difference in turbidity at 650 nm compared with controls without GRX3. These results show that GRX1 can be reduced by GSH and clearly displays a disulfide reductase activity, whereas GRX3 appears unable to reduce insulin disulfides.
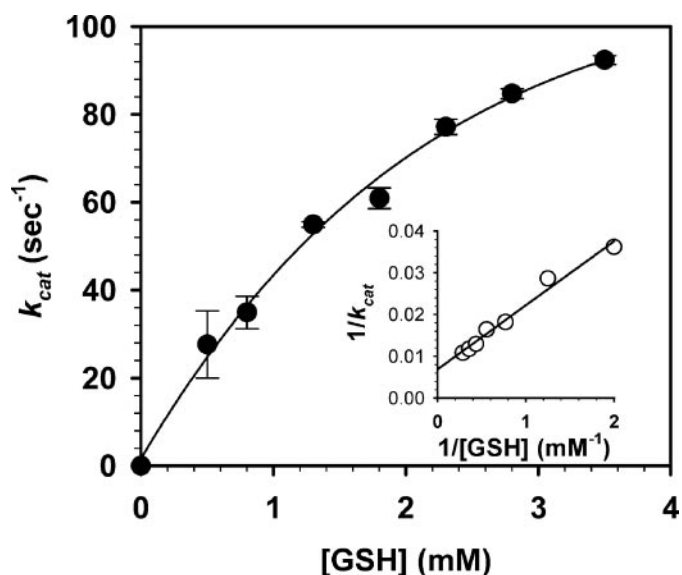
GRX Activity as a GSH-disulfide Oxidoreductase (HED Assay)—In the HED assay, the classic assay to test deglutathionylation activity, glutaredoxin activity is measured by following NADPH oxidation at 340 nm in a coupled system with 1 mM GSH and glutathione reductase. In this assay, glutaredoxin catalyzes the reduction of the mixed disulfide that is spontaneously formed between HED and GSH during the time of preincubation in the absence of glutaredoxin. The kinetic parameters were determined at pH 7.9, taking into account the background due to the spontaneous reaction between HED and GSH that leads to increased NADPH consumption. For this reason, we used GSH concentrations up to 3.5 mM. The results of these experiments demonstrate that GRX1 catalyzed the reduction of the glutathionylated substrate with an apparent K_m for GSH of ∼2.65 ± 0.51 mM and a turnover number of 161.6 ± 15 s^{-1} (Fig. 3 and Table 2). Both kinetic values are within the range determined for other GRXs (42, 43). As in the case of the DHAR and disulfide reductase activities, no HED activity could be detected for CrGRX3.

Reduction of Glutathionylated A_4-GAPDH by TRXh1, GRX1, and GRX3—We have recently demonstrated that A_4-GAPDH activity is reversibly inhibited by glutathionylation (11). This protein can be used as a more physiological substrate to test deglutathionylation activity. To obtain glutathionylated A_4-GAPDH, the protein was incubated in the presence of 0.1 mM H_2O_2 plus 0.5 mM GSH. After 15-min incubation, BPGA, the substrate of GAPDH, was added. This treatment allowed protection of residual GAPDH activity from any further inactivation, because BPGA forms a stable thioester with the catalytic cysteine but had no reactivation effect (Fig. 4*A*). Subsequently, the recovery of GAPDH activity was evaluated using either the NADPH-thioredoxin system or the GSH-glutaredoxin system. As shown in Fig. 4*B*, GAPDH activity was restored to ∼55% by 5 μM GRX1 in the presence of 5 mM GSH, whereas GSH alone was totally ineffective (Fig. 4*A*). A similar reactivation was
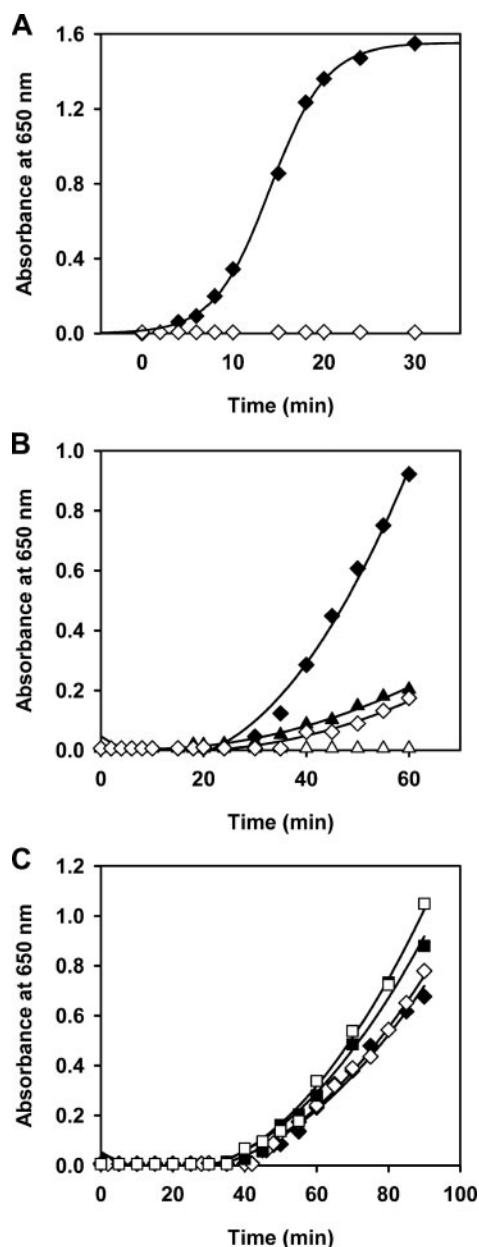
FIGURE 2. **Insulin disulfide reductase activity of *Chlamydomonas* TRX*h*1, GRX1, and GRX3.** The rate of insulin reduction was assessed by measuring the turbidity at 650 nm in reaction assays containing 0.33 mM DTT or 1 mM GSH in the presence of 5 μM of each enzyme: *A*, TRX*h*1 at pH 7.0 (*black diamonds*); the negative control (DTT alone) is represented by *open diamonds*. *B*, GRX1 in the presence of DTT (*black diamonds*) or GSH (*black triangles*) at pH 7.0; negative controls are represented by *open diamonds* (DTT alone) and *open triangles* (GSH alone). *C*, GRX3 in the presence of DTT at pH 7.0 (*black diamonds*) or at pH 7.9 (*black squares*); negative controls are represented by *open diamonds* (DTT alone at pH 7.0) and *open squares* (DTT alone at pH 7.9). Please note that the *x*-axis in *panel C* is different from those in *panels A* and *B*. Three separate measurements were made for each protein, and the mean is shown. The standard deviations were omitted for clarity.

obtained with 100 μM TRX*h*1 in the presence of NADPH and NADPH-thioredoxin reductase (Fig. 4*B*). When we compared the percentage of reactivation, the recovery of GAPDH activity obtained by the two systems did not reach the levels obtained in the presence of DTT (~90%). This can be explained by the fact that H_2O_2 plus GSH leads to either glutathionylation or primary oxidation of catalytic cysteine to sulfenic acid, the latter

FIGURE 3. **Relative activity of *Chlamydomonas* GRX1 with GSH in the GSH-disulfide oxidoreductase assay (HED assay).** Variations of the turnover number during HED assay catalyzed by 10 nM GRX1 in the presence of varying GSH concentrations. Turnover represents moles of NADPH oxidized/sec by 1 mol of GRX1. Activity was calculated after subtracting the spontaneous reduction rate observed in the absence of GRX1. Michaelis-Menten and Lineweaver-Burk (*inset*) plots of k_{cat} *versus* [GSH] are shown. The conditions of the assay (with 0.7 mM HED) are described under "Experimental Procedures." Three separate experiments were performed, and the data are represented as mean $\pm$ S.D. The kinetic parameters were calculated using only nonlinear curve fit of the data sets.

TABLE 2

Glutathione-dependent reduction of the mixed disulfide formed between glutathione and hydroxyethyl disulfide by *Chlamydomonas* GRX1

The reaction was performed at pH 7.9 in a mixture containing glutathione reductase, NADPH, 0.7 mM HED, and 0.5–3.5 mM GSH. For more details see "Experimental Procedures." Kinetic parameters were calculated by non-linear regression using the Michaelis-Menten equation. Data shown are mean $\pm$ S.D.

	K_m	k_{cat}	k_{cat}/K_m
	mM	s^{-1}	$M^{-1}s^{-1}$
GRX1	2.65 ± 0.51	161.6 ± 15	6.1×10^4

being more efficiently reduced by 20 mM DTT than by the two enzymatic systems (11).

The kinetic parameters of these deglutathionylation reactions were determined for GRX1 and TRX*h*1 (Fig. 5). The values obtained showed that GRX1, with an $S_{0.5}$ below 1 μM, is a very efficient catalyst of deglutathionylation, whereas TRX*h*1 appears as a poor reductant of glutathionylated GAPDH with an $S_{0.5} \sim$ 40-fold higher than the value measured for GRX1 (Table 3). These results indicate that GRX is a specific enzyme involved in the deglutathionylation mechanism, as previously reported (18). On the other hand, GRX3 was not able to restore GAPDH activity in the presence of GSH (Fig. 4*B*). However, pre-reduced GRX3, prepared by DTT reduction and subsequent dialysis to remove DTT, was able to reactivate GAPDH when used in a molar excess with respect to the glutathionylated enzyme (10:1 molar ratio) (Fig. 4*B*). The percentage of reactivation was 55% as in the case of GRX1 and TRX*h*1. This result suggests that, as previously reported for yeast GRX5 (14) and *E. coli* GRX4 (31), *Chlamydomonas* GRX3 may not be reduced by GSH. This lack of reduction would explain the
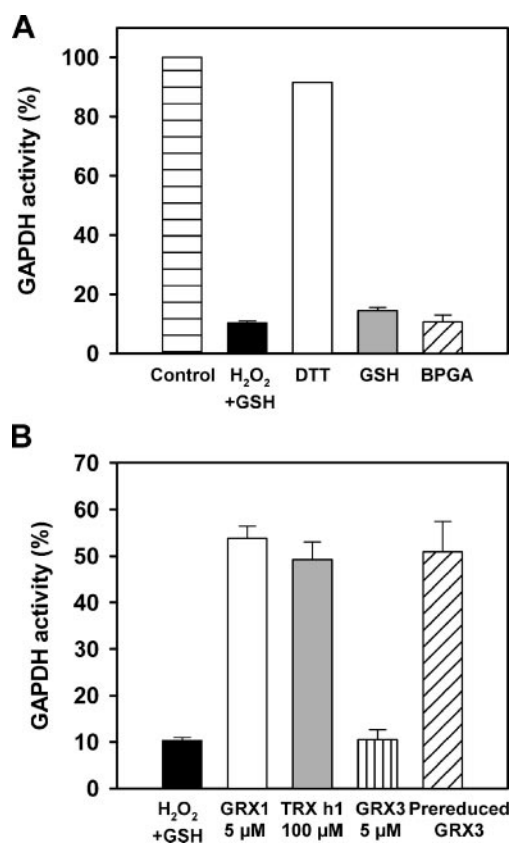
FIGURE 4. **Reactivation of glutathionylated A$_4$-GAPDH.** *A*, reactivation by DTT, GSH, and BPGA. A$_4$-GAPDH was inactivated by incubation with 0.1 mM H$_2$O$_2$ in the presence of 0.5 mM GSH for 15 min at 25 °C and subsequently treated with 20 mM DTT (*white bar*), or 5 mM GSH (*gray bar*), or 35 μM BPGA (*diagonal bar*) for 10 min at 25 °C. *B*, reactivation of glutathionylated A$_4$-GAPDH by GRX1, TRXh1, and GRX3. A$_4$-GAPDH was inactivated by incubation with 0.1 mM H$_2$O$_2$ in the presence of 0.5 mM GSH for 15 min at 25 °C and subsequently treated with 35 μM BPGA. The reactivation was performed in the presence of 5 μM GRX1 plus 5 mM GSH (*white bar*), or 100 μM TRXh1 plus 0.3 mM NADPH and 0.2 μM NADPH-thioredoxin reductase (*gray bar*), or 5 μM GRX3 plus 5 mM GSH (*vertical bar*), or 20 μM of DTT-treated GRX3 (*diagonal bar*). The NADPH-dependent activity was determined before (*black bar*) and after the different treatments. Activities are represented as a percentage of the initial activity measured before the inactivation treatment (*control bar*). The data are shown as mean ± S.D.

absence of activity of GRX3 in the HED and DHAR assays. To determine whether GRX3 disulfide bond could be reduced by GSH, oxidized GRX3 was incubated with 5 mM GSH, and the number of free thiols was determined. As expected, no reduction was observed as the number of free thiols remained unchanged after GSH treatment (0.28 ± 0.02). This indicates that GRX3 is not reduced by GSH. This lack of reduction by GSH prompted us to determine the redox potential of GRX3 disulfide bond.

Determination of the Redox Potential of GRX3—To determine the redox potential of GRX3, redox titrations were performed in the presence of DTNB as a probe to reveal free protein thiols under varying redox conditions. As described under "Experimental Procedures," the enzyme was incubated with different ratios of reduced and oxidized DTT. Subsequently, the excess of DTT was removed by desalting, and the number of free thiols was measured using DTNB. Data from redox titrations of GRX3 were fitted to a Nernst equation for one disulfide-dithiol interchange. At pH 7.9, the standard redox poten-
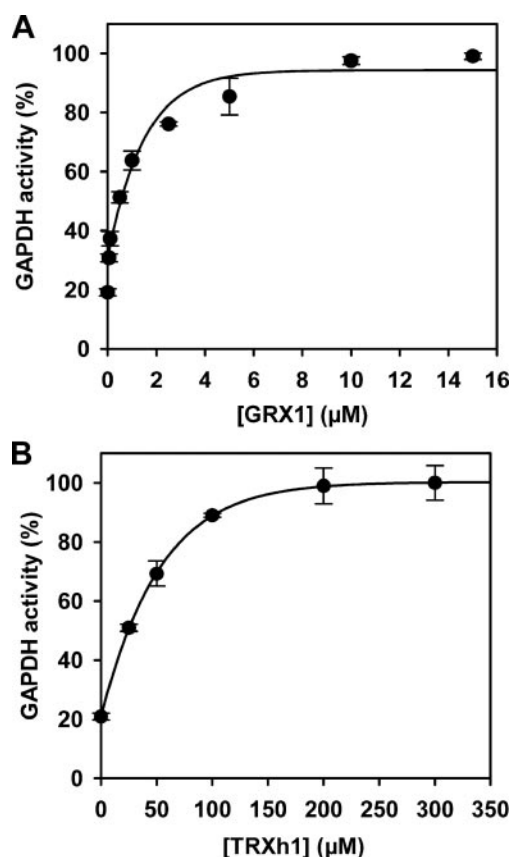
FIGURE 5. **Activities of GSH-GRX1 and NADP-TRXh1 systems with glutathionylated A$_4$-GAPDH.** Reactivation of glutathionylated A$_4$-GAPDH was performed under the following conditions: *A*, 5 mM GSH in the presence of varying concentrations of GRX1 ranging from 0.05 to 15 μM; *B*, 0.3 mM NADPH, 0.2 μM NTR in the presence of varying concentrations of TRXh1 ranging from 25 to 300 μM. Activities are represented as a percentage of the maximal activity measured for each reactivation system. Data are shown as a mean ± S.D.

TABLE 3

Activation parameters of glutathionylated A$_4$-GAPDH by *Chlamydomonas* GRX1, TRXh1, and GRX3

The $S_{0.5}$ values were determined by varying concentrations from 0.05 to 15 μM for GRX1, from 25 to 300 μM for TRXh1, and from 2.5 to 30 μM for GRX3. The standard deviations were calculated from three independent experiments. $t_{1/2}$ values correspond to the time of preincubation necessary to reach half-maximal reactivation of glutathionylated A$_4$-GAPDH at a concentration of GRX1, TRXh1, and GRX3 corresponding to the $S_{0.5}$ value.

Protein	$S_{0.5}$	$t_{1/2}$
	μM	min
GRX1[a]	0.87 ± 0.13	1–2
TRXh1[b]	37.7 ± 2.5	>10
GRX3[c]	4.54 ± 0.37	2–3

[a] Varying concentration of GRX1 in the presence of 5 mM GSH.
[b] Varying concentrations of Trxh1 in the presence of 0.3 mM NADPH and 0.2 μM NTR.
[c] Varying concentration of GRX3 in the presence of the thylakoid-dependent light activation system as described in a previous study (9).

tial ($E_{m,7.9}$) of GRX3 disulfide was −323 ± 4 mV (Fig. 6). This value places GRX3 in a position much closer to plastidial thioredoxins than to other GRXs (40, 44, 45). Indeed, thioredoxins exhibit redox potentials in the range of −335 to −357 mV (at pH 7.9), while glutaredoxins have redox potentials ranging from −244 to −284 mV (at pH 7.9) (17). This unusually low redox potential of GRX3, close to that of chloroplastic TRXs, prompted us to examine whether GRX3 might be reduced in
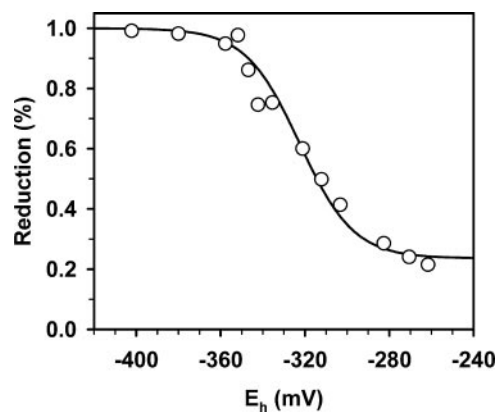
FIGURE 6. **Redox titration of *Chlamydomonas* GRX3.** The percentage of reduction was determined by DTNB. The number of reacting thiols of GRX3 was measured after a 3-h incubation at 25 °C with 20 mM DTT in various dithiol-disulfide ratios. For more details see the "Experimental Procedures." Results were fitted by non-linear regression to the Nernst equation for one redox component. Data are represented as mean ± S.D. of triplicate determinations.

FIGURE 7. **Reactivation of glutathionylated A$_4$-GAPDH by GRX3.** A$_4$-GAPDH was inactivated by incubation with 0.1 mM H$_2$O$_2$ in the presence of 0.5 mM GSH for 15 min at 25 °C and subsequently treated with 35 μM BPGA. The *arrow* indicates that the inactivated enzyme was submitted to various reactivation treatments. The reactivation was performed in the presence of the reconstituted thylakoid-dependent light activation system (*LS, white bar*), or LS without FTR in the presence of 15 μM GRX3 (*gray bar*), or LS complete in the presence of 15 μM GRX3 (*diagonal bar*). The NADPH-dependent activity was determined before (*black bar*) and after the different treatments. Activities are represented as a percentage of the initial activity measured before the inactivation treatment. Data are shown as a mean ± S.D.

FIGURE 8. **Activities of GRX3 with glutathionylated A$_4$-GAPDH in the presence of the reconstituted thylakoid-dependent light activation system.** Reactivation of glutathionylated A$_4$-GAPDH was performed with the reconstituted thylakoid-dependent light activation system in the presence of varying concentrations of GRX3 ranging from 2.5 to 30 μM. Activities are represented as a percentage of the maximal activity measured with this reactivation system. Data are shown as a mean ± S.D.

the light by the ferredoxin and ferredoxin-thioredoxin reductase, the chloroplast thioredoxin reduction system.

Chlamydomonas GRX3 Is a Substrate for Ferredoxin-Thioredoxin Reductase—To test whether GRX3 could be a substrate for ferredoxin-thioredoxin reductase (FTR), we measured the reactivation of glutathionylated GAPDH by GRX3 in the light in the presence of the reconstituted thylakoid-dependent reduction system composed of purified thylakoids, ferredoxin, and ferredoxin-thioredoxin reductase. Surprisingly, GRX3 was able to restore GAPDH activity in the light (Fig. 7). The extent of reactivation was comparable to that obtained with the GSH-GRX1 and NADPH-TRXh1 systems (Fig. 4*A*). This reactivation was only observed when the thylakoid-dependent system was complete. In fact, when we omitted FTR or GRX3, no reactivation was detected (Fig. 7). The kinetic parameters of this reaction indicate that, with an $S_{0.5}$ of 4.54 ± 0.37 μM, and a $t_{1/2}$ estimated to be between 2 and 3 min (Table 3), GRX3 is very efficiently reduced by FTR, at least as efficiently as chloroplastic TRX (Fig. 8 and Table 3) (33). In contrast, no reactivation of A$_4$-GAPDH could be observed in the light in the presence of the reconstituted thylakoid-dependent reduction system and the four types of chloroplastic TRX: f, m, x, and y (data not shown). Similarly, the recently described chloroplastic NADPH thioredoxin reductase C protein (46) from *Arabidopsis* appeared unable to efficiently reduce GRX3 (data not shown). The efficient reactivation of GAPDH by GRX3 demonstrates that GRX3, using electrons from light via FTR, reduces glutathionylated substrates such as chloroplastic A$_4$-GAPDH.

DISCUSSION

The characterization of *Chlamydomonas* GRX1 and GRX3 reported here reveals that the two enzymes exhibit very different biochemical properties. Cytosolic GRX1 is a typical CPYC-type GRX, which exhibits DHAR, disulfide reductase, and deglutathionylation activities. The kinetic parameters measured for these activities are comparable to those previously reported for other CPYC-type GRX from *E. coli* or human (15, 16, 42, 43). In contrast, chloroplastic GRX3 is a CGFS-type
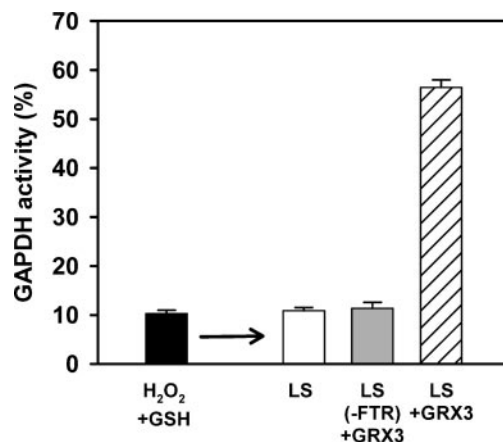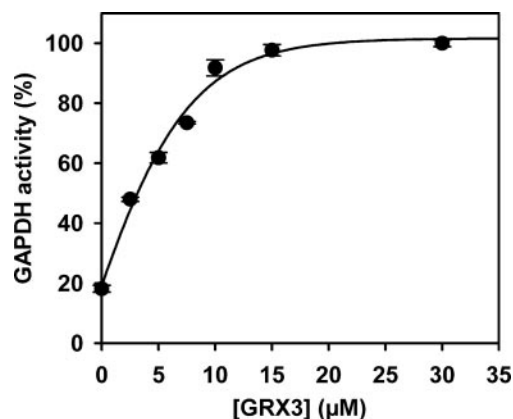
GRX, which has very different and unexpected biochemical properties. First, GRX3 is not reduced by GSH as previously observed for CGFS-type GRX from yeast (14) and *E. coli* (31). This inability to be reduced by GSH might thus be a general property of CGFS-type GRX and could explain the lack of activity of GRX3 in the DHAR and HED assays but not in the insulin assay, because, even in the presence of DTT, no disulfide reductase activity could be measured as previously reported for *E. coli* GRX4 (31). This absence of disulfide-oxidoreductase activity is not likely to reflect a higher substrate specificity, because the reduction of insulin is considered as a nonspecific disulfide reductase assay. Additional biochemical analyses on other GRX will be required to determine whether the absence of disulfide reductase activity might be common to all CGFS-type GRXs.
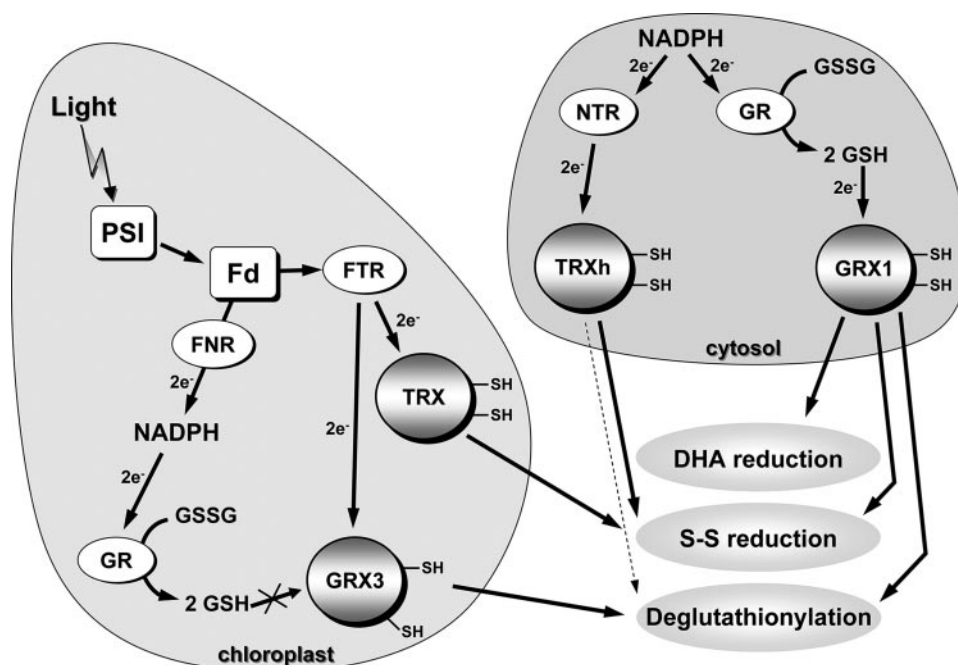
FIGURE 9. **Thioredoxin and glutaredoxin redox network in *Chlamydomonas*.** In the cytosol, CPYC-type glutaredoxin (*GRX1*) is reduced by reduced glutathione (*GSH*) generated from oxidized glutathione (*GSSG*) by glutathione reductase (*GR*) which uses electrons provided by NADPH. GRX1 can catalyze DHA reduction and disulfide reduction and also appears to be very efficient in catalyzing A_4-GAPDH deglutathionylation. Cytosolic thioredoxin (*TRX*) h1 is a very efficient disulfide oxidoreductase but is a very poor catalyst of protein deglutathionylation. In the chloroplast, TRXs f, m, x, and y are reduced in the light by photosystem I (*PSI*) photoreduced ferredoxin (*Fd*) and FTR. These TRXs can reduce very efficiently disulfide bonds on diverse target proteins (Lemaire *et al.* 50) but are inefficient in catalyzing protein deglutathionylation. In chloroplasts, GSSG is reduced by GR using electrons provided by the NADPH pool, itself reduced by Fd and Fd-NADP reductase (*FNR*) in the light. However, glutathione cannot reduce the CGFS-type GRX3, the major chloroplastic GRX in *Chlamydomonas*. Instead, GRX3 is reduced in the light through Fd and FTR. This chloroplastic GRX exhibits neither disulfide reductase nor DHAR activities but catalyzes very efficiently protein deglutathionylation.

FTR can use a substrate distinct from TRX. This result is consistent with the fact that CGFS-type GRX4 from *E. coli* was previously shown to be reduced by *E. coli* thioredoxin reductase, although less efficiently (31). In the case of GRX3, the $S_{0.5}$ was below 5 μM (Table 3) despite the production of oxygen by the thylakoid reduction system in the samples during the course of the experiments. This value indicates that GRX3 is more efficiently reduced by FTR than some chloroplastic TRX and suggests that this reduction of GRX3 by FTR may be physiological.

The ability of GRX1 and GRX3 to catalyze protein deglutathionylation was measured using glutathionylated A_4-GAPDH as a substrate. This chloroplastic GAPDH was recently shown to undergo glutathionylation with a concomitant loss of enzyme activity (11). The glutathionylated residue being most likely the catalytic cysteine, the reactivation of the glutathionylated enzyme is strictly correlated with its glutathionylation status. The redox and structural properties of chloroplastic A_4-GAPDH closely resembles that of cytosolic glycolytic GAPDH, which also undergoes inactivation by glutathionylation (47–49). Therefore, A_4-GAPDH, whose activity is easily measurable, constitutes a good model substrate to compare the efficiency of diverse cytosolic and chloroplastic oxidoreductases in catalyzing protein deglutathionylation.

Another unexpected property of GRX3 is its atypically negative redox potential of -323 ± 4 mV at pH 7.9, the physiological pH of the chloroplast stroma in the light. This value is much closer to the redox potentials classically reported for TRXs than GRXs (40, 44, 45). To our knowledge, GRX3 has the most negative redox potential ever measured for a GRX. However, the absence of reduction of GRX3 by GSH is not likely to be linked to this low redox potential. Indeed, this lack of reduction could more likely be accounted for by a problem of interaction between GRX3 and GSH, because some of the activity assays were performed in the presence of glutathione reductase, which allows decreasing the redox potential of a GSH solution below the redox potential of GRX3. The redox potential of another CGFS-type GRX, yeast mitochondrial GRX5, has been previously reported to be -175 mV (14). The huge difference with the value reported here for *Chlamydomonas* GRX3 may be linked to the methods employed to estimate the redox potential. Alternatively, it could reflect differences of CGFS-type GRX redox properties between species or between subcellular compartments. It may also be linked to the fact that the two proteins use different reductants or reductases with distinct redox properties. The low redox potential of chloroplastic GRX3, close to that of TRXs, prompted us to evaluate whether FTR, the chloroplastic thioredoxin reductase, could be able to reduce GRX3. The results indicate that GRX3 is very efficiently reduced by FTR in the light. It is the first study reporting that

Both *Chlamydomonas* GRX1 and GRX3 were found to catalyze A_4-GAPDH deglutathionylation very efficiently, whereas TRXs, either from the cytosol (h1) or the chloroplast (f, m, x, and y), appeared poorly or not efficient at all. For example, cytosolic GRX1 appears 40-fold more efficient than cytosolic TRXh1, the best thioredoxin tested (Table 3). Moreover, the $S_{0.5}$ value of 38 μM measured for TRXh1 does not seem to be physiological. These results are consistent with previous studies that suggested that GRXs are much more efficient than TRXs to catalyze protein deglutathionylation (18, 19). Therefore, despite the greater diversity of TRX isoforms in photosynthetic organisms, GRXs appear as the only known enzymes able to control protein glutathionylation. GRX3 is the first chloroplastic enzyme shown to catalyze protein deglutathionylation. This is especially important, because *Chlamydomonas* chloroplasts do not apparently contain typical CPYC GRX (21). Moreover, A_4-GAPDH is the first physiological substrate identified for a CGFS-type GRX. Indeed, the physiological substrates of yeast GRX5 (14) or *E. coli* GRX4 (31) remain unknown.

The results reported here provide new insights into the complex redox regulatory network present in photosynthetic orga-

nisms (Fig. 9). In chloroplasts, several enzymes important for photosynthetic carbon assimilation through the Calvin cycle have been shown to undergo glutathionylation, such as TRXf (9), fructose-1,6-bisphosphate aldolase (5), or A_4-GAPDH (11). It has been proposed that glutathionylation may constitute a new mechanism of regulation of the Calvin cycle (50). To date, GRX3 is the only enzyme reported to control deglutathionylation in the chloroplast and might therefore play a major role in the regulation of photosynthetic metabolism and especially the Calvin cycle under conditions of oxidative stress. Further studies are required to determine the functional importance of GRX3 *in vivo* as well as the biochemical properties and the role of other GRX isoforms present in photosynthetic organisms.

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**Biochemical Characterization of Glutaredoxins from *Chlamydomonas reinhardtii*
Reveals the Unique Properties of a Chloroplastic CGFS-type Glutaredoxin**

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Lemaire

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