

# Glutathionylation in the Photosynthetic Model Organism *Chlamydomonas reinhardtii*: A Proteomic Survey\*<sup>§</sup>

Mirko Zaffagnini<sup>‡§</sup>, Mariette Bedhomme<sup>‡¶</sup>, Hayam Groni<sup>||</sup>, Christophe H. Marchand<sup>‡||</sup>, Carine Puppo<sup>\*\*</sup>, Brigitte Gontero<sup>\*\*</sup>, Corinne Cassier-Chauvat<sup>‡‡</sup>, Paulette Decottignies<sup>||§§</sup>, and Stéphane D. Lemaire<sup>‡¶¶</sup>

**Protein glutathionylation is a redox post-translational modification occurring under oxidative stress conditions and playing a major role in cell regulation and signaling. This modification has been mainly studied in nonphotosynthetic organisms, whereas much less is known in photosynthetic organisms despite their important exposure to oxidative stress caused by changes in environmental conditions. We report a large scale proteomic analysis using biotinylated glutathione and streptavidin affinity chromatography that allowed identification of 225 glutathionylated proteins in the eukaryotic unicellular green alga *Chlamydomonas reinhardtii*. Moreover, 56 sites of glutathionylation were also identified after peptide affinity purification and tandem mass spectrometry. The targets identified belong to a wide range of biological processes and pathways, among which the Calvin-Benson cycle appears to be a major target. The glutathionylation of four enzymes of this cycle, phosphoribulokinase, glyceraldehyde-3-phosphate dehydrogenase, ribose-5-phosphate isomerase, and phosphoglycerate kinase was confirmed by Western blot and activity measurements. The results suggest that glutathionylation could constitute a major mechanism of regulation of the Calvin-Benson cycle under oxidative stress conditions. *Molecular & Cellular Proteomics* 11: 10.1074/mcp.M111.014142, 1–15, 2012.**

From the <sup>‡</sup>Laboratoire de Biologie Moléculaire et Cellulaire des Eucaryotes, FRE3354 Centre National de la Recherche Scientifique, Université Pierre et Marie Curie, Institut de Biologie Physico-Chimique, 75005 Paris, France, the <sup>||</sup>Institut de Biochimie et Biophysique Moléculaire et Cellulaire, UMR8619 Centre National de la Recherche Scientifique, Université Paris-Sud, 91405 Orsay Cedex, France, the <sup>\*\*</sup>Laboratoire d'Enzymologie de Complexes Supramoléculaires, Bioénergétique et Ingénierie des Protéines, UPR9036 Centre National de la Recherche Scientifique, Institut de Microbiologie de la Méditerranée-Aix-Marseille Universités, BP71, 13402 Marseille Cedex 20, France, and the <sup>‡‡</sup>Laboratoire de Biologie Intégrative, URA2096 Centre National de la Recherche Scientifique, Commissariat à l'Energie Atomique-Saclay, Institut de Biologie et de Technologies de Saclay, Service de Biologie Intégrative et Génétique Moléculaire, 91191 Gif sur Yvette Cedex, France

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Protein post-translational modifications (PTMs)<sup>1</sup> play a pivotal role in cellular signaling (1). Recently, redox PTMs have emerged as important mechanisms of signaling and regulation in all organisms. Our increasing understanding of the molecular mechanism of cell signaling has revealed that reactive oxygen species (ROS) and reactive nitrogen species (RNS) act as signaling molecules to transfer extracellular or intracellular information and elicit specific responses. ROS and RNS have generally been considered to be toxic molecules that have to be continuously scavenged and efficiently detoxified. Plant cells exhibit a remarkable ability to cope with high rates of ROS/RNS production as a result of a complex scavenging system that includes either antioxidant molecules or enzymes (2). In photosynthetic organisms, ROS and RNS are continuously produced during normal aerobic metabolism but are also produced transiently in response to various types of endogenous or exogenous signals, such as biotic and abiotic stresses. This production activates specific signaling pathways, resulting in transcriptional, post-transcriptional and post-translational responses that will, *in fine*, allow adaptation to new environmental conditions. These past decades, redox modifications have emerged as central mechanisms in these processes, at the interface between ROS/RNS and the adaptive responses to environmental changes.

ROS/RNS signaling operates mainly through a set of PTMs of thiol residues on proteins (3). Indeed, cysteine residues can undergo different states of oxidation such as sulfenic, sulfinic, and sulfonic acids in addition to protein disulfide bridges (intra- or intermolecular), S-thiolation (mainly glutathionylation), or nitrosylation. Small disulfide oxidoreductases named thioredoxins (TRXs) and glutaredoxins play a prominent role in the control of most of these modifications that can affect the

<sup>1</sup> The abbreviations used are: PTM, post-translational modification; BioGEE, *N*-biotinyl glutathione ethyl-ester; BioGSSG, *N,N'*-biotinyl glutathione disulfide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, reduced glutathione; GSSG, glutathione disulfide; IAM, iodoacetamide; LC, liquid chromatography; PGK, phosphoglycerate kinase; PRK, phosphoribulokinase; RNS, reactive nitrogen species; ROS, reactive oxygen species; RPI, ribose-5-phosphate isomerase; TRX, thioredoxin; TAP, Tris acetate phosphate; BPGA, 1,3-bisphosphoglycerate.

function of numerous proteins by modifying their activity, their subcellular localization, their stability, or their interactions with partner proteins.

Glutathione serves as one of the major cellular antioxidant redox buffers. It is a highly abundant tripeptide ( $\gamma$ -L-glutamyl-L-cysteinyl-L-glycine) present at millimolar concentrations in many subcellular compartments. Glutathione occurs mostly under the reduced form (GSH) because oxidized glutathione (GSSG) is continuously regenerated into GSH by glutathione reductase using NADPH as electron donor. Glutathione has numerous documented physiological functions. It is generally considered to constitute a redox buffer and also participates in the detoxification of ROS, heavy metals (through phytochelatins), and xenobiotics (through glutathione S-transferases) (2, 4). In addition to these functions, glutathione can form a mixed disulfide bridge between the thiol group of its cysteine and an accessible free thiol on a protein, a reaction termed protein S-glutathionylation. This PTM can protect specific cysteine residues from irreversible oxidation but can also modulate protein activities. The exact mechanism(s) leading to protein glutathionylation *in vivo* remain(s) unclear, whereas the reverse reaction, named deglutathionylation, is likely catalyzed by glutaredoxins, proteins belonging to the TRX family. Glutathionylation appears to play a major role in numerous fundamental cell processes and is implicated in a broad spectrum of human diseases including cancer, diabetes, and several neurodegenerative, cardiovascular, or pulmonary diseases (5, 6). Moreover, there is also a strong interplay between glutathionylation and other redox PTMs (7), especially nitrosylation as recently illustrated by the demonstration that the activity of endothelial nitric oxide synthase is regulated by glutathionylation (8).

To date, glutathionylation has been generally studied in nonphotosynthetic organisms where proteomic studies, mainly based on the use of [ $^{35}$ S]cysteine labeling, have allowed identification of nearly 200 targets involved in diverse cell processes (5, 9–12). Very recently, a large scale analysis in *Plasmodium falciparum* identified 493 putative targets of glutathionylation (13). Although the number of studies on glutathionylation in plants remains limited (14), several plant enzymes have been shown to undergo glutathionylation including TRXs (15, 16), protein tyrosine phosphatase 1B (17), glyceraldehyde-3-phosphate dehydrogenase (18, 19), isocitrate lyase (20), galactono- $\gamma$ -lactone dehydrogenase (21), glycine decarboxylase (22), peroxiredoxins (23, 24), and methionine sulfoxide reductase (25).

Only three proteomic studies aimed at analyzing the diversity of glutathionylated proteins in photosynthetic organisms have been reported. Two studies on *Arabidopsis* using biotinylated glutathione identified two (26) or 79 (27) glutathionylated proteins. In the unicellular eukaryotic green alga *Chlamydomonas reinhardtii*, a major photosynthetic model organism, [ $^{35}$ S]cysteine labeling allowed 25 glutathionylated proteins to be identified (23).

Here we report a large scale proteomic analysis that allowed identification of 225 glutathionylated proteins in *C. reinhardtii* using biotinylated glutathione and streptavidin affinity chromatography. Moreover, the sites of glutathionylation (cysteine residues) were also identified after peptide affinity purification and tandem mass spectrometry. The identified targets belong to a wide range of biological processes and pathways, among which the Calvin-Benson cycle appears as a major target with 10 of 11 enzymes identified. The glutathionylation of four of these enzymes was confirmed by Western blot and activity measurements. Our results strongly suggest that glutathionylation could constitute a major mechanism of regulation of the Calvin-Benson cycle under oxidative stress conditions.

### MATERIALS AND METHODS

**Materials and Enzymes**—Modified trypsin was obtained from Promega (Madison, WI). EZ-Link Sulfo-NHS-Biotin was from Perbio Science (Cramlington, UK). NAP-5 columns and 5,5'-dithiobis-2-nitrobenzoic acid were purchased from GE Healthcare and Pierce, respectively. Biotinylated glutathione ethyl ester (BioGEE) was obtained from Invitrogen. High performance liquid chromatography grade ethanol and acetic acid were purchased from VWR France, and all of the other reagents were from Sigma-Aldrich.

**Chlamydomonas Cultures and Protein Extraction**—The *C. reinhardtii* D66 cell wall-less strain was grown in Tris acetate phosphate (TAP) medium (28) at 25 °C under constant agitation and continuous light ( $100 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ). Two liters of *Chlamydomonas* culture at  $5\text{--}7 \times 10^6$  cells/ml were harvested by centrifugation and resuspended in 2 ml of 30 mM Tris-HCl, pH 7.9, 1 mM EDTA, supplemented with 100  $\mu\text{M}$  PMSF and a mixture of protease inhibitors (Complete, Mini, EDTA-free; Roche Applied Science). Total soluble proteins were extracted by two freeze/thaw cycles in liquid nitrogen, and protein concentrations were determined by the Bradford assay using bovine serum albumin as a standard (29).

**Synthesis of BioGSSG**—EZ-Link Sulfo-NHS-Biotin, a soluble biotinylation reagent, was used to couple biotin to the primary amino group of oxidized glutathione (GSSG) under mild alkaline conditions. The biotinylation reagent (50  $\mu\text{l}$ , 64 mM) was added to GSSG (50  $\mu\text{l}$ , 32 mM) in 100 mM potassium phosphate buffer, pH 8.0, and the mixture was left for 1 h at room temperature. After incubation, unreacted biotin was quenched by adding 35  $\mu\text{l}$  of 0.6 M ammonium bicarbonate ( $\text{NH}_4\text{HCO}_3$ ).

To assess the efficiency of the synthesis, reverse phase high performance liquid chromatography analysis using an Alliance system (Waters, UK) equipped with a diode array detector showed a major and a minor chromatographic peaks. Each peak was collected manually, and the identities of the products were determined by MALDI-TOF-MS (Voyager-DE STR; Perseptive Biosystems, Framingham, MA) and ESI-IT-MS/MS (Agilent Technologies, Santa Clara, CA). MS and MS/MS spectra (data not shown) revealed that the compound present in the major peak had a mass of 1065.4 Da corresponding to *N,N'*-biotinyl glutathione disulfide, named BioGSSG for brevity, formed by the addition of one biotin moiety on each amino group of  $\gamma$ -Glu residues and that the compound present in the minor peak had a mass of 839.3 Da corresponding to *N*-biotinyl glutathione disulfide (the addition of one biotin moiety on one of the two amino groups of  $\gamma$ -Glu residues).

**Synthesis of BioGEE**—BioGEE was either purchased directly from Invitrogen or synthesized from glutathione ethyl ester in the laboratory, as follows. EZ-Link Sulfo-NHS-Biotin, a soluble biotinylation

reagent, was used to couple biotin to the primary amino group of glutathione ethyl ester. The biotinylation reagent (300  $\mu$ l, 25 mM) was added to glutathione ethyl ester (300  $\mu$ l, 25 mM) in 50 mM potassium phosphate buffer, pH 7.2, and the mixture was left to derivatize for 1 h at room temperature. After incubation, unreacted biotin was quenched by adding 82.5  $\mu$ l of 0.6 M ammonium bicarbonate buffer. The concentration of BioGEE was determined spectrophotometrically with 5,5'-dithiobis-2-nitrobenzoic acid using a molar extinction coefficient at 412 nm of 14,150  $\text{M}^{-1} \text{cm}^{-1}$  to calculate the free thiol content of BioGEE (30).

**Detection of Glutathionylation in Vivo**—A *Chlamydomonas* D66 cell culture was grown in 100 ml of TAP medium to  $6\text{--}8 \times 10^6$  cells/ml. After centrifugation ( $4,000 \times g$ , 3 min), the pellet was washed once with fresh TAP medium and resuspended to  $8 \times 10^6$  cells/ml in TAP medium supplemented with BioGEE or BioGSSG (final concentration, 0.5–2 mM). After different incubation times (30 min to 2 h) to allow entry of biotinylated glutathione, 0.5 or 1 mM  $\text{H}_2\text{O}_2$  or diamide was added for various times (from 5 to 60 min) with or without DMSO to force BioGSSG entry, either in the light or in the dark. After treatment, the medium was discarded by centrifugation, and cells were washed twice with TAP medium to remove the biotinylated glutathione not conjugated with proteins and resuspended in 30 mM Tris-HCl, pH 7.9. Total soluble proteins were then extracted by two freeze/thaw cycles in liquid nitrogen, and protein concentration was determined as described above. Protein extracts were loaded on gradient (4–12%) SDS-polyacrylamide gel and analyzed by Western blot using anti-biotin antibodies as described previously (20). As control, a protein extract was treated for 15 min with 20 mM reduced DTT before loading to assess the reversibility of the reaction.

**Glutathionylation of Protein Extracts**—Freshly prepared protein extracts (3.4 mg/ml) were incubated in 100 mM Tris-HCl, pH 7.9, in the presence of various concentrations of BioGSSG ranging from 0 to 5 mM. Control samples were incubated with 100 mM iodoacetamide (IAM) for 30 min in the dark prior to incubation in the presence of 2 mM BioGSSG. After 1 h of incubation, BioGSSG-treated samples were alkylated in the presence of 100 mM IAM or treated with 20 mM reduced DTT for 15 min to assess the reversibility of the reaction. All of the treatments were performed at room temperature.

**SDS-PAGE and Western Blotting**—BioGSSG-treated samples were loaded on SDS-polyacrylamide gel electrophoresis using the Bio-Rad Mini Protean system. Sixteen  $\mu$ l of samples in SDS sample buffer without any reducing agent were resolved by 10% SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membranes using a Bio-Rad semidry blotter. Signals corresponding to glutathionylated proteins tagged with biotin were visualized by Western blot using anti-biotin antibodies, as described previously (20).

**Purification of Glutathionylated Proteins**—Freshly prepared protein extracts (12 mg/ml) were incubated with 2 mM BioGSSG for 1 h at room temperature in a final volume of 400  $\mu$ l. A control experiment was performed without BioGSSG. After incubation, the excess of BioGSSG was removed by a single passage on G-25 gel filtration columns (HiTrap desalting, 5 ml; GE Healthcare). Desalted samples were then loaded on streptavidin-agarose columns (1 ml) pre-equilibrated with 30 mM Tris-HCl, pH 7.9, and 150 mM NaCl. Subsequently, the column was extensively washed with 10 ml of washing buffer (30 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.25% Triton X-100 plus 600 mM NaCl) and with 2 ml of washing buffer without NaCl. Finally, bound proteins were eluted with 20 mM DTT in 10 mM ammonium bicarbonate ( $\text{NH}_4\text{HCO}_3$ ). The eluted proteins were concentrated by evaporation using a vacuum concentrator and separated on a reducing 10% SDS-PAGE gel and stained with Coomassie Brilliant Blue R-250. Visible protein bands were excised manually and then submitted to destaining and in-gel digestion with modified porcine trypsin using

the automated system Digest Pro96 (Intavis AG, Bremen, Germany) as previously described (31). The peptides were dissolved in 18  $\mu$ l of 3% acetonitrile ( $\text{CH}_3\text{CN}$ ) containing 0.1% v/v formic acid and analyzed by mass spectrometry.

**Tandem Mass Spectrometry**—NanoLC-CHIP-IT-MS/MS experiments were performed on an Agilent 1200 nanoflow LC system coupled to a 6330 Ion Trap equipped with the Chip Cube orthogonal ionization system (Agilent Technologies, Santa Clara, CA) as previously described (32). Briefly, peptide samples (4  $\mu$ l) were loaded at a flow of 4  $\mu$ l/min onto the enrichment column equilibrated in 3%  $\text{CH}_3\text{CN}$  in 0.1% formic acid. Then the peptides were eluted toward the analytical column with a constant flow of 300 nl/min. The gradient profile consisted in two linear gradients (3–20%  $\text{CH}_3\text{CN}$  in 0.1% formic acid over 3 min and 20–50%  $\text{CH}_3\text{CN}$  in 0.1% formic acid over 12 min) followed by an isocratic step at 70%  $\text{CH}_3\text{CN}$  in 0.1% formic acid for 5 min and an equilibration step with 3%  $\text{CH}_3\text{CN}$  in 0.1% formic acid for 7 min. Five scans were averaged to obtain a full scan spectrum from which the four most abundant ions (singly charged ions excluded) were selected for fragmentation by collision-induced dissociation. For each fragmentation spectrum, four scans were averaged. All of the spectra were saved in profile mode, and other ion trap parameters were set as follows: capillary, –2000 V; skimmer, 30 V; Cap exit, 100 V; fragmentation amplitude, 1.30 V; smart parameter settings, on; drying gas, nitrogen at 300 °C with a flow rate of 4 liters/min. Helium was used as cooling and fragmentation gas at a pressure of  $6 \times 10^{-7}$  mbar.

**Purification of Glutathionylated Peptides**—After glutathionylation and G-25 gel filtration steps, the proteins were concentrated up to 8–10 mg/ml using Microcon devices YM-10 (Millipore). 65  $\mu$ g of proteins were denatured with the Protein extraction Reagent-4 kit (Sigma) 10 min at room temperature and then alkylated with 15 mM IAM for 90 min in the dark. IAM concentration was lowered by three times dilution in 65 mM ammonium bicarbonate, and protein extract was loaded on immobilized trypsin column (Trypsin spin column from Sigma, prepared following the manufacturer's recommendations) for 30 min at room temperature. The peptides were eluted with 100 mM ammonium bicarbonate. Eluted peptides were loaded onto a 1-ml streptavidin-agarose column and eluted with DTT following the procedure described above. Eluted peptides were then concentrated up to 50  $\mu$ l by evaporation using a vacuum concentrator and analyzed by tandem mass spectrometry as described above by injecting increasing amounts of peptides. Each analysis was performed in duplicate.

**Data Analysis and Database Search**—Raw MS/MS data were processed by Data Analysis software version 3.4 (Agilent Technologies, Santa Clara, CA). For protein and peptide identifications, files corresponding to control or glutathionylation conditions were concatenated into a single file for further use with Mascot MS/MS Ions Search and Phenyx.

For identifications with Mascot, the NCBI nr database (release 20091202, 10,107,245 entries) was reduced to the *Viridiplantae* taxonomy. Mass accuracy tolerance was set to 100 ppm on the parent ion mass and 0.6 Da in MS/MS mode. One missed cleavage per peptide was allowed. No molecular weight or isoelectric point restriction were selected. Only high quality mass spectra (individual ion score above identity threshold *i.e.* 40 for a *p* value < 0.05) were taken into account to identify glutathionylated sites or glutathionylated proteins.

For identifications with Phenyx, a sequence database restricted to *C. reinhardtii* (15,006 entries) was uploaded in Fasta format from the Uniprot release 15.15 (March 2, 2010) database. The parameters selected for scoring model and parent charge were ESI ion trap and (two or three, trust = yes), respectively. Parent tolerance was set to 100 ppm, and methionine oxidation was considered as variable modification. Acceptance thresholds were set as follow: peptide length  $\geq$



6; peptide z-score  $\geq 6$ ; peptide  $p$  value  $\leq 10^{-4}$  with a global AC score  $\geq 6$  (for identification of glutathionylated sites) or 12 (for identification of glutathionylated proteins). Because two sets of calculations have been carried out, the first round was more stringent (enzyme: trypsin KR\_noP; missed cleavage  $\leq 1$ ; cleavage mode: normal) than the second (enzyme: trypsin KR; missed cleavage  $\leq 2$ ; cleavage mode: semi-tryptic).

For protein identifications with the two search engines, methionine oxidation and cysteine carboxyamidomethylation were taken into account as variable modifications. For identification of glutathionylated peptides, only methionine oxidation was selected as variable modification. Glutathionylated proteins were validated if they were identified by both programs with at least two different peptides meeting acceptance criteria, whereas glutathionylated cysteine-containing peptides proposed by both Mascot and Phenix software were only considered as identified after manual validation of the corresponding MS/MS spectra.

**Consensus Sequence Analysis**—For each putative site of glutathionylation (SSG-modified sites) 10 residues on each side of the glutathionylated cysteine were selected. Frequencies of flanking residues were computed with motif-x software (33). For predictions of secondary structure and solvent accessibility, the NetSurfP server was employed (34).

**Plasmid Construction for Expression of RPI and PGK in *Escherichia coli***—The cDNA clones encoding RPI (AV390085) and PGK (BP097682) from *C. reinhardtii* were obtained from the Kazusa DNA Research Institute (Chiba, Japan). The coding sequences were amplified by PCR, and specific restriction sites (underlined) were introduced at the 5' and 3'-ends of the coding sequences using the following specific primers: 5' NcoI-RPI, CGC**ACCATGG**CCGCGCCG-GTCTCAA; 3' BamHI-RPI, GCACG**GGATCC**TTAGTGCTTCTTGGGG-TTGGG; 5' NdeI-PGK, CATCGT**CATATG**GCGGTGAAGAAGTCGG-TTGG; and 3' BamHI-PGK, ATTGCG**GGATCC**TACTTCTCGTCCAG-GGCGG. After purification, the PCR products were digested by NcoI/NdeI and BamHI and cloned into the NcoI/NdeI and BamHI restriction sites of modified pET-3c-His (NdeI) or pET-3d-His (NcoI) vectors (Novagen) allowing expression of the proteins with a polyhistidine tag at the N terminus. All of these expression vectors were verified by DNA sequencing.

**Protein Purification**—Glyceraldehyde-3-phosphate dehydrogenase ( $A_4$ -GAPDH) was purified from *Chlamydomonas* as previously described (35). Recombinant proteins (RPI and PGK) were produced using the pET vectors/BL21 expression system. Bacteria were grown in LB medium supplemented with 100  $\mu$ g/ml ampicillin at 37 °C until the absorbance at 600 nm reached 0.6. The expression of RPI or PGK was then induced by transfer to 27 °C for 18 h with (RPI) or without (PGK) the addition of 200  $\mu$ M isopropyl- $\beta$ -D-thiogalactopyranoside. The cells were harvested by centrifugation, resuspended in 30 mM Tris-HCl, pH 7.9, 10  $\mu$ g/ml PMSF, and broken using a French press ( $7 \times 10^7$  Pa), and cell debris was removed by centrifugation. The supernatants containing the soluble His-tagged proteins were loaded onto a Ni<sup>2+</sup> 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 using imidazole step gradients according to the manufacturer's instructions. The molecular mass and purity of the recombinant proteins were checked by SDS-PAGE after dialysis against 30 mM Tris-HCl, pH 7.9, 1 mM EDTA. The protein concentrations were determined spectrophotometrically using calculated molar extinction coefficient at 280 nm (14,690  $M^{-1}\cdot cm^{-1}$  and 20,065  $M^{-1}\cdot cm^{-1}$  for RPI and PGK, respectively). The resulting homogeneous proteins were stored at -20 °C.

**Enzyme Assays**—For phosphoribulokinase activity assays, total protein extracts from *Chlamydomonas* (0.16 mg/ml) were treated with 10 mM DTT, and the excess of reducing agent was removed by

desalting in 100 mM Tris-HCl, pH 7.9, using NAP-5 columns. The eluates were then incubated in the presence of 5 mM GSSG, and PRK activity was monitored as described in Ref. 36. For the analysis of *Chlamydomonas*  $A_4$ -GAPDH, incubations were performed as described previously (19). Briefly, the enzyme (2.5  $\mu$ M) was incubated in 50 mM Tris-HCl, pH 7.9, and 0.2 mM NADP<sup>+</sup> at room temperature in the presence of H<sub>2</sub>O<sub>2</sub> and GSH at the indicated concentration. When indicated, BPGA (5 units/ml of yeast 3-phosphoglycerate kinase, 2 mM ATP, and 3 mM 3-phosphoglycerate) was incubated with the enzyme for 5 min prior to inactivation treatments. Reactivation was performed by adding 20 mM DTT to the treated samples. At the indicated times, the aliquots were withdrawn to assay enzyme activity monitored as described previously (19).

**In Vitro Glutathionylation of RPI, PGK, and  $A_4$ -GAPDH Using BioGSSG**—Before BioGSSG treatments, all of the proteins were reduced with 10 mM DTT and desalted in 100 mM Tris-HCl, pH 7.9, using NAP-5 columns. The pre-reduced proteins were incubated in 100 mM Tris-HCl, pH 7.9, in the presence of 2 mM BioGSSG. Control samples were incubated with 100 mM IAM for 30 min in the dark prior to incubation in the presence of 2 mM BioGSSG. After 1 h of incubation, BioGSSG-treated samples were alkylated in the presence of 100 mM IAM or treated with 20 mM DTT for 30 min to assess the reversibility of the reaction. All of the treatments were performed at room temperature. Proteins were then loaded on nonreducing SDS-PAGE and analyzed by Western blotting using anti-biotin antibodies as described previously (20).

## RESULTS

**Detection of S-Glutathionylated Proteins in *Chlamydomonas***—Initially, we focused on the identification of proteins undergoing glutathionylation *in vivo*. For this purpose, protein glutathionylation was analyzed in *Chlamydomonas* cell cultures treated with different biotinylated forms of glutathione: BioGSSG or BioGEE, and under various culture conditions as described under "Materials and Methods." Although some glutathionylated proteins have been occasionally detected during these experiments, the efficiency of the labeling was unsatisfactory, and this method was not further considered for identifying glutathionylated proteins.

Therefore the detection of S-glutathionylated proteins was performed with *Chlamydomonas* cell extracts treated with BioGSSG. This molecule promotes protein glutathionylation by itself via thiol-disulfide exchange reactions and allows visualization of glutathionylated proteins by immunoblotting using anti-biotin antibodies (Fig. 1, procedure 2). As shown in Fig. 2, numerous proteins were labeled after treatment with BioGSSG, and the signals increased following a dose-dependent profile relative to increasing concentrations of BioGSSG (0.1–5 mM) (Fig. 2). In the control sample (absence of BioGSSG) two prominent bands of ~35 and ~70 kDa were observed that likely represent endogenously biotinylated proteins.

The extent of protein S-glutathionylation after treatment with 2 mM BioGSSG was strongly diminished by a pretreatment with the thiolate alkylating agent IAM (Fig. 2). This is consistent with the fact that IAM pretreatment blocks free accessible cysteines and confirms that the BioGSSG labeling is dependent on the presence of reactive cysteines on proteins.

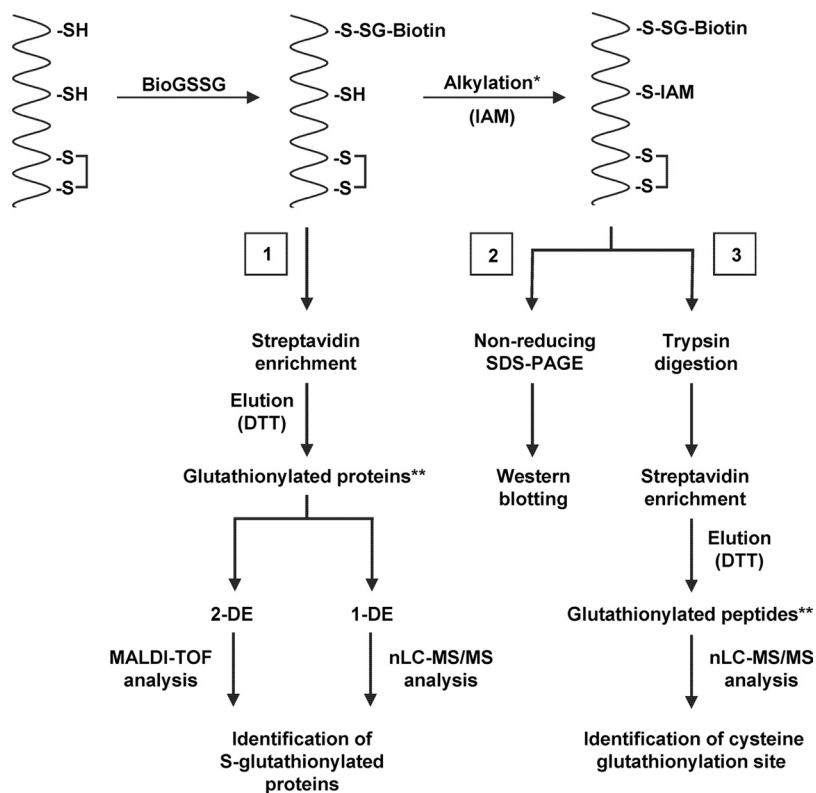


FIG. 1. Schematic diagram of the methods employed to study protein glutathionylation in the present study. Procedure 1, work flow for identification of putative glutathionylated targets. Procedure 2, Western blot using anti-biotin antibodies. Procedure 3, work flow for identification of S-glutathionylation sites (cysteines) on proteins. \*, alkylation during procedure 3 was performed under denaturing conditions. \*\*, proteins and peptides initially glutathionylated with BioGSSG.

BioGSSG	0	0.1	0.5	2	5	2	0	2	2	(mM)
IAM	-	-	-	-	-	+	-	-	+	
DTT	-	-	-	-	-	-	+	+	+	

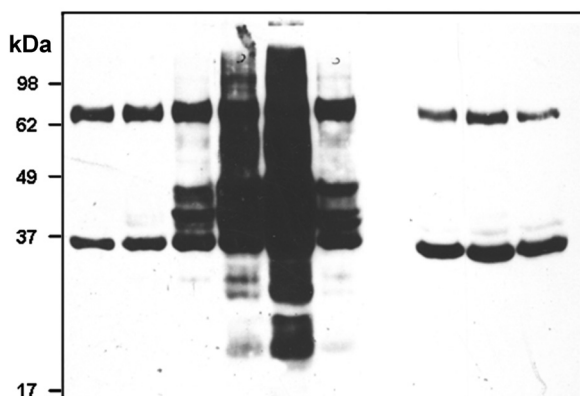
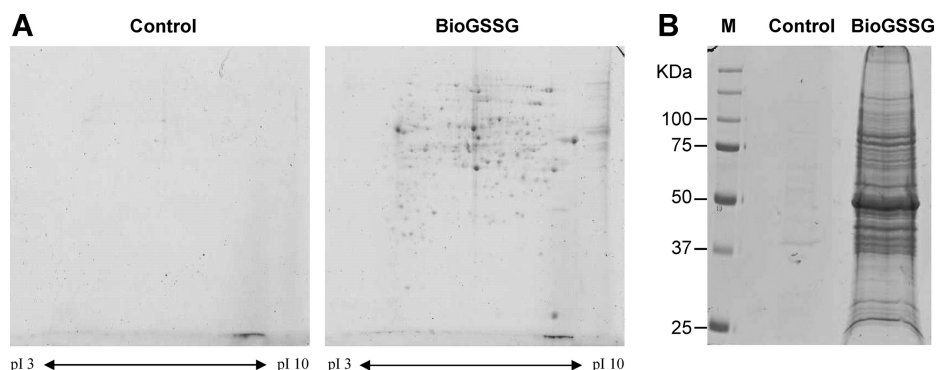


FIG. 2. Western blot probed with anti-biotin antibodies to detect S-glutathionylated proteins in protein extract from *Chlamydomonas* following BioGSSG treatment. The figure shows a concentration-dependent increase in S-glutathionylation following a 1-h incubation in the presence of BioGSSG (0–5 mM). Two endogenous biotin-reactive bands at ~35 and 70 kDa remain constant throughout and fortuitously serve as loading controls. A control sample was incubated with 100 mM IAM for 30 min in the dark followed by a 1-h incubation in the presence of 2 mM BioGSSG. The reversibility of the reaction was assessed by treating control, BioGSSG-treated, and IAM/BioGSSG-treated samples with 20 mM DTT for 30 min as indicated. The proteins were resolved by nonreducing SDS-PAGE and transferred to nitrocellulose for Western blotting with anti-biotin antibodies as described under “Materials and Methods.”

Signals generated by BioGSSG at 2 mM were abolished by treatment with the reducing agent DTT, indicating that these signals indeed correspond to glutathionylated proteins (Fig. 2). In fact, the reducing treatment also removed the signals in

the BioGSSG-treated sample preincubated with IAM. This indicates that the signals remaining even after IAM treatment likely correspond to unblocked cysteines rather than to aspecific reaction of BioGSSG with noncysteine amino acids. On



**FIG. 3. Coomassie-stained two- and one-dimensional gels of S-glutathionylated proteins purified by streptavidin-agarose affinity chromatography.** *A* and *B*, Coomassie-stained S-glutathionylated proteins from *Chlamydomonas* protein extracts after two-dimensional (*A*) or one-dimensional electrophoresis (*B*). Proteins labeled with BioGSSG were purified as described under “Materials and Methods.” Control gel (*A*) and control lane (*B*) correspond to the protein extract submitted to the procedure of streptavidin-agarose affinity chromatography but omitting the initial BioGSSG treatment. For two-dimensional gels, the proteins were separated based on pI (3–10 linear gradient, *left to right*), and molecular mass (12% SDS-PAGE, top to bottom), whereas for one-dimensional gels, the proteins were separated based on molecular mass (12% SDS-PAGE).

the contrary, the two bands present in all samples, including in the control sample not treated with BioGSSG, did not disappear after DTT treatment. Therefore, these bands likely correspond to proteins carrying a biotin molecule as biotin decarboxylases, where biotin is covalently bound to the  $\epsilon$ -amino group of lysine (37, 38).

Overall, these results demonstrate the efficiency and specificity of BioGSSG for detection of S-glutathionylated proteins in *Chlamydomonas* extracts. Because numerous bands could be detected, this prompted us to purify and identify the corresponding proteins.

**Identification of Glutathionylated Proteins in *Chlamydomonas* Cell Extracts**—*Chlamydomonas* cell extracts were treated for 1 h with BioGSSG, and taking advantage of the biotin moieties, S-glutathionylated proteins were purified using affinity chromatography on a streptavidin-agarose matrix (Fig. 1, procedure 1). Proteins bound to the matrix by association with the biotinylated glutathione tag were selectively released using DTT to cleave the mixed disulfides and then analyzed by two-dimensional gel electrophoresis (Fig. 3A).

In the eluate derived from BioGSSG treatment, ~52 spots could be detected, after Coomassie Blue staining, in the pH range between 3 and 10. The visible spots were excised from the gel and digested with trypsin. The resulting peptides were subjected to MALDI-TOF mass spectrometry, allowing identification of 34 proteins by peptide mass fingerprinting (supplemental Table S1, proteins marked in the 2D column). Very few spots were visible in the control sample corresponding to the DTT eluate from a streptavidin column loaded with a protein extract not treated with BioGSSG. This indicates that the procedure allows identification of glutathionylated proteins with high specificity.

To increase the diversity of identified glutathionylated proteins, BioGSSG-treated extracts were affinity-purified as above, and the eluate was separated by one-dimensional gel

electrophoresis and subjected to nanoLC-MS/MS (Fig. 3B). As observed previously, very few proteins were detected in the control sample, whereas the eluate derived from BioGSSG treatment contained numerous proteins. All of the protein bands were analyzed by nanoLC-MS/MS, allowing identification of 208 proteins (supplemental Table S1). Each assignment was based on data submitted to two different search engines for two distinct peptides for which a high degree of certainty was achieved ( $p < 0.05$ ). A similar analysis performed on the control sample identified few peptides belonging to only four very abundant *Chlamydomonas* proteins (Rubisco, elongation factor 1 $\alpha$ , tubulin  $\alpha$ , and enolase). These four proteins were also identified in the treated sample but with numerous peptides and high sequence coverage. This indicates that they are likely retained on the column after BioGSSG treatment because they are glutathionylated and that their presence as traces in the control sample likely reflects their abundance. Among all of the proteins identified in the BioGSSG-treated sample, only two lack cysteines, but the sequences of these predicted proteins are probably partial because they are both translated from genome sequences containing several gaps. The full-length sequence of these proteins may therefore contain one or several cysteine residues.

Among the 208 proteins identified by nanoLC-MS/MS, 31 of 34 proteins identified by two-dimensional gel electrophoresis and MALDI-TOF MS could be recovered. Overall, the two approaches employed (MALDI-TOF and nanoLC-MS/MS) allowed identification of 211 potential targets of protein glutathionylation in *C. reinhardtii*.

Most of the proteins identified were never found previously as potential candidates for glutathionylation in plant cells, including a number of miscellaneous and hypothetical proteins. The 211 identified proteins are involved in numerous cell processes and metabolic pathways (Fig. 4). Many proteins

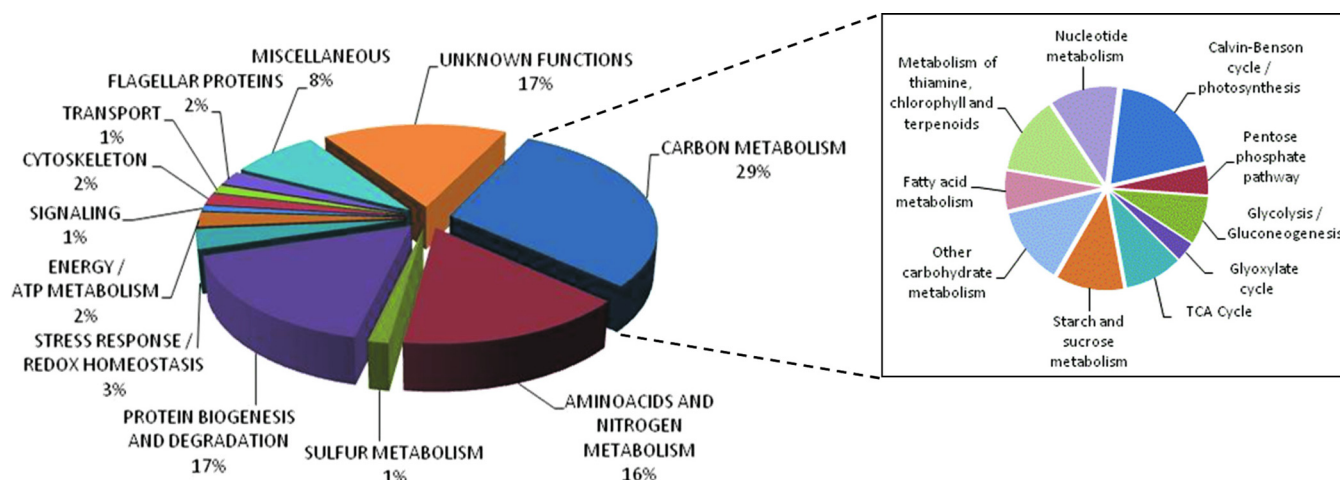


FIG. 4. Functional categories of S-glutathionylated proteins identified using BioGSSG in *Chlamydomonas* cell extract. The pie chart represents the functional categories of S-glutathionylated proteins identified in *C. reinhardtii* cell extracts after BioGSSG treatment. The prominent category, carbon metabolism, is further detailed in the right panel.

participate in amino acid and nitrogen metabolism (17%) and protein biogenesis and degradation (17%), but the most prominent category is carbon metabolism (29%), which accounts for nearly one-third of the proteins identified. This category can be further divided in subcategories, thereby revealing that the process containing the highest number of putative targets of glutathionylation is photosynthesis and especially CO<sub>2</sub> assimilation (Fig. 4). Indeed, 10 of 11 enzymes of the Calvin-Benson cycle were identified (supplemental Table S1). Overall, these results suggest that, as in mammals, numerous proteins can undergo glutathionylation the green alga *C. reinhardtii*. This strengthens the potential importance of glutathionylation in plants and its role in the redox control of numerous cell processes and metabolic pathways.

**Identification of S-Glutathionylation Sites**—Concomitantly to the identification of proteins undergoing S-glutathionylation, the procedure described above was adapted to enable unbiased specification of SSG-cysteine residues on proteins. Because the BioGSSG-based method allowed capture and identification of S-glutathionylated proteins, we reasoned that it could similarly be used to capture SSG-cysteine containing peptides in tryptic digests of S-glutathionylated proteins. Then for peptides with a suitable mass to charge ratio, it should be possible to use tandem mass spectrometry to elucidate the peptide sequence and identify both the protein of origin and the site of S-glutathionylation. To this end, the scheme depicted in Fig. 1 (procedure 3) was applied to isolate and analyze S-glutathionylated peptides by nanoLC-MS/MS.

*Chlamydomonas* extracts were treated for 1 h with BioGSSG followed by alkylation with IAM under denaturing conditions to block completely all remaining free cysteine thiols as done previously for affinity purification of S-glutathionylated proteins. At this stage, instead of capturing intact S-glutathionylated proteins on streptavidin-agarose, the proteins were subjected to complete trypsinolysis. Glu-

thionylated peptides were affinity-purified on streptavidin agarose, followed by selective cleavage of the disulfide bond linking the peptide cysteine and its biotinylated glutathione tag (using 20 mM DTT). This purification procedure based on selective peptide capture allows unequivocal identification of the target cysteine, not only when one cysteine is present in the eluted peptide (unmodified cysteine in the eluate) but also when the peptide contains several cysteines. Indeed, because of the IAM alkylation before trypsinolysis, when a peptide containing several cysteine residues is eluted from the column, the cysteine originally glutathionylated is unmodified, whereas the other cysteines are carbamidomethylated. Moreover, because the elution of peptides is performed under gentle conditions and follows extensive washing steps, one would not predict significant contamination by peptides that bind streptavidin-agarose nonspecifically as observed in the elution profile of control samples not treated with BioGSSG (Fig. 3).

The eluted peptides were analyzed by nanoLC-MS/MS, and a total of 71 different peptides were identified. A total of 12 peptides were excluded: 2 peptides identified in the control, 4 false positive peptides without cysteine, and 5 that could not be validated because of poor fragmentation spectra. The remaining 59 peptides correspond to 41 different proteins (Table I). Many peptides belong to proteins already identified above under native conditions, but several sites were also identified in new targets. This method allowed identification of 56 putative glutathionylated cysteines: 39 sites in 27 proteins among the 211 putative targets previously identified and 17 sites in 14 new putative target proteins (Table I). Interestingly, many of these new potential targets of glutathionylation correspond to proteins belonging to high molecular weight complexes, such as ribosomal proteins. This suggests that some of these glutathionylation sites might be shielded under native conditions and therefore not retained on streptavidin column.



## Glutathionylation Targets in Chlamydomonas

TABLE I  
Glutathionylated peptides identified in *C. reinhardtii*

RefSeq, accession number in NCBI database. The glutathionylated cysteine-containing peptide column shows the primary sequences of glutathionylated peptides; the positions of modified cysteines in the full-length sequences of the corresponding proteins are indicated as exponents. Column # shows protein identified as glutathionylated by BioGSSG (this work). Column S shows proteins identified as *in vivo* targets of S-thiolation in *C. reinhardtii* by [<sup>35</sup>S]cysteine labeling (23). Column T shows proteins identified as thioredoxin targets in *C. reinhardtii* (55).

Protein name	RefSeq	#	S	T	Glutathionylated cysteine-containing peptide
Carbon metabolism (17 proteins)					
Calvin-Benson cycle/photosynthesis					
Apoferredoxin 1	XP_001692808			×	K.TPSGDKTIEC <sup>48</sup> PADTYILDAAEEAGLDLPHYSC <sup>69</sup> R.A
Fructose-1,6-bisphosphatase	XP_001690872	×			K.GTIDGELATVISSVSLAC <sup>109</sup> K.Q
Plastocyanin	XP_001702952				A.GEYGYVC <sup>130</sup> EPHQGAGMVGK.I K.LTAAGEYGYVC <sup>130</sup> EPHQGAGMVGK.I
Phosphoglycerate kinase	XP_001699523	×	×		K.GC <sup>411</sup> ITTIIGGDSVAAVEQAGVAEK.M K.VDDC <sup>458</sup> IGPEVEK.A
Phosphoribulokinase	XP_001694038	×		×	K.TVVIGLAADSGC <sup>47</sup> GK.S K.MFDPVYLFDEGSTISWIPC <sup>274</sup> GR.K
Ribulose biphosphate carboxylase large chain	NP_958405	×		×	N.ATAGTC <sup>247</sup> EEMMKR.A R.GLLGC <sup>172</sup> TIKPK.L R.VALEAC <sup>427</sup> TQAR.N
Transketolase	XP_001701881	×			K.SGHPGMPMGC <sup>84</sup> APMGYVLLWNEVMK.Y
Gluconeogenesis, glycolysis					
Enolase	XP_001702971	×		×	K.KVEEVLNLC <sup>23</sup> VK.E K.LTTENIC <sup>357</sup> QVVGDDILVTNPVR.V
Fructose-1,6-bisphosphate aldolase	XP_001700659	×		×	R.GILAMDESNAIC <sup>58</sup> GK.R R.GILAMDESNAIC <sup>58</sup> GK.L K.VMFEGLLLKPAVTPGADC <sup>256</sup> K.N K.VMFEGLLLKPAVTPGADC <sup>256</sup> KNK.A K.NVVLLAC <sup>405</sup> DAFGALPPVSR.L
Phosphoenolpyruvate carboxykinase, splice variant	XP_001694964	×			
Glyoxylate cycle					
Isocitrate lyase	XP_001695331	×	×	×	R.TAEGFYC <sup>247</sup> VR.G
Malate synthase	XP_001695632	×			K.C <sup>79</sup> APPAPGLVDR.R
TCA cycle					
Aconitate hydratase, mitochondrial	XP_001689702	×		×	K.AGLIGSC <sup>400</sup> TNSSYEDMAR.A
Starch and sucrose metabolism					
ADP-glucose pyrophosphorylase large subunit	XP_001693447	×			R.LIDVPMSC <sup>112</sup> INSGISK.I
Phosphorylase, plastidial	XP_001700091				R.LAAC <sup>171</sup> FLDSMATLPLPGWGYGIR.Y
Metabolism of thiamine, chlorophyll, and terpenoids					
Full-length thiazole biosynthetic enzyme	XP_001698672	×		×	K.DMDEFAESDVVIVGAGSAGLAC <sup>106</sup> AFELGR.I
Magnesium-chelatase subunit chl, chloroplastic	XP_001691232	×	×		K.KIPMVDLPLGATEDRVC <sup>184</sup> GTIDIEK.A
Amino acids and nitrogen metabolism (3 proteins)					
Acetohydroxy acid isomeroreductase	XP_001702649	×		×	K.GHPFSEIC <sup>439</sup> NESIIEAVDSLNPYMHAR.G
Adenosylhomocysteinase	XP_001693339	×			K.DIAEADFGRLIEDLAEAEPMGLMAC <sup>41</sup> R.S R.LEIDLAEAEPMGLMAC <sup>41</sup> R.S K.SKFDNVYGC <sup>243</sup> R.H R.MNEPEVC <sup>444</sup> VSTTNR.N
Isopropylmalate dehydratase, large subunit	XP_001702135	×			
Protein biogenesis and degradation (11 proteins)					
Protein biosynthesis, translation					
Eukaryotic translation elongation factor 1 $\alpha$ 1	XP_001696568	×		×	K.EHLSIVIC <sup>13</sup> GHVDSGK.S K.NMISGAAQADV <sup>112</sup> LLMVPADGNFTTAIQK.G R.VEQGVVVKPGDEVIFLPTHHTANPC <sup>303</sup> TGK.V R.LC <sup>728</sup> EPVYLVEIQAPEALGGIYSTLNTK.R
Elongation factor 2	XP_001703215	×		×	R.HYAHVDC <sup>82</sup> PGHADYVK.N
Elongation factor Tu	XP_001696344	×	×	×	H.VDC <sup>82</sup> PGHADYVK.N R.AGQGAFGNM <sup>96</sup> R.G R.SAALINQSC <sup>165</sup> HVR.V R.C <sup>101</sup> REEFLTR.R K.TIAEC <sup>161</sup> LADELVNAAK.G R.GC <sup>102</sup> IVSPDLAVLNLVIVK.K R.KVVC <sup>115</sup> SC <sup>117</sup> AVITDYGETAGLSMLQEYK.L
Ribosomal protein L4	XP_001694804				
Ribosomal protein L9	XP_001700532				
Ribosomal protein L21	XP_001698024				
Ribosomal protein S5	XP_001695400				
Ribosomal protein S6	XP_001695102				
40 S ribosomal protein S12	XP_001698669				
Folding/molecular chaperones					
Chaperonin 60B2	XP_001692504	×			R.C <sup>537</sup> SLENAVSVAK.T R.MLAEYENC <sup>249</sup> R.I
Degradation					
E3 ubiquitin ligase	XP_001690964				K.GLLDLTC <sup>115</sup> QTVAQMIK.G
Energy/ATP metabolism (1 protein)					
Vacuolar ATP synthase subunit E	XP_001692936	×		×	R.INC <sup>200</sup> SNTLDDR.L



TABLE I—continued

Protein name	RefSeq	#	S	T	Glutathionylated cysteine-containing peptide
Cytoskeleton (3 proteins)					
Actin	XP_001699068	×		×	K. EKL <sup>C219</sup> YVALDFEQEMATALSSSALEK.T
Tubulin $\alpha$ 1/ $\alpha$ 2 chain	XP_001691876	×			R. AVC <sup>376</sup> MISNSTAIGEIFSR.L
	XP_001703110				R. C <sup>65</sup> IFLDLEPTVVDEVR.T
Tubulin $\beta$ 1/ $\beta$ 2 chain	XP_001693997	×			R. EIVHIQGGQC <sup>12</sup> GNQIGAK.F
	XP_001694072				K. NMMC <sup>301</sup> AADPR.H
Miscellaneous (2 proteins)					
Inosine-5'-monophosphate dehydrogenase	XP_001699045				R. GFTSV <sup>C138</sup> VTDTGALGGK.L
Low CO <sub>2</sub> inducible protein LCIB	XP_001698344	×			K. C <sup>320</sup> YTVVNLK.T
Unknown functions (4 proteins)					
Predicted protein	XP_001696868				K. AAELEAVGSAVC <sup>109</sup> AK.K
					K. SC <sup>248</sup> LGIVDNTGK.L
Predicted protein	XP_001694293				R. VEVQDTSGGC <sup>30</sup> GAMYR.I
Predicted protein	XP_001698018	×			R. NPDKC <sup>38</sup> AALAAEGAK.V
Predicted protein	XP_001691572				K. AGGVVEAAC <sup>154</sup> VIELPFLK.G

By contrast, these sites could have been exposed after trypsinolysis, allowing the purification and identification of the corresponding peptides. If we include these new proteins, a total of 225 potential glutathionylated proteins were identified using BioGSSG. Analysis of the primary amino acid sequence surrounding the 56 putative sites of glutathionylation did not reveal any consensus sequence motifs (data not shown). Further analysis of the secondary structure immediately surrounding modified cysteines indicated that SSG sites have a random distribution within  $\alpha$ -helical,  $\beta$ -strand, or coil structures (data not shown). The structural determinants underlying the receptive nature of these glutathionylation sites are therefore most probably dependent on the microenvironment of the cysteine residue within the three-dimensional structure of the protein.

**Calvin-Benson Cycle Enzymes as Major Targets of S-Glutathionylation**—The Calvin-Benson cycle appears to be a major target of glutathionylation in *Chlamydomonas* with 10 of 11 enzymes of this pathway identified using BioGSSG (supplemental Table S1). Only triose phosphate isomerase has not been found but was identified as a target of glutathionylation in an earlier study in *Arabidopsis* (26). These results are consistent with the previous identification in *Chlamydomonas*, using an *in vivo* proteomic approach based on [<sup>35</sup>S]cysteine radiolabeling (23), of two Calvin-Benson cycle enzymes: RPI and PGK. They are also consistent with the fact that all Calvin-Benson cycle enzymes were previously suggested to be redox-regulated (39).

The glutathionylation of several Calvin-Benson cycle enzymes was further investigated at the biochemical level. We focused on four enzymes, namely PRK, PGK, RPI, and A<sub>4</sub>-GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Because PRK activity is restricted to the Calvin-Benson cycle enzyme, the effect of glutathionylation treatments on the enzyme activity was analyzed in total protein extracts of *Chlamydomonas*. After incubation with 5 mM GSSG, a classical *in vitro* glutathionylation test, PRK activity was found to decrease to ~15% of its initial activity (Fig. 5). This inactivation was reversed in the presence of DTT as expected for a mod-

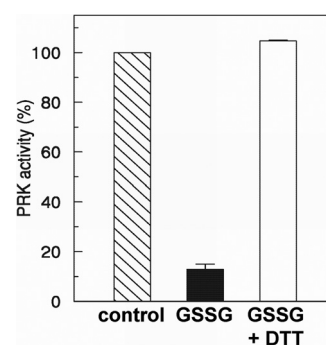


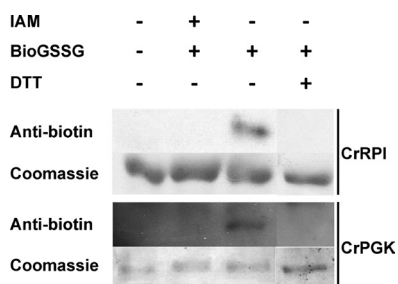
FIG. 5. **Effect of GSSG on PRK activity.** Protein extract of *Chlamydomonas* cell culture was treated with 5 mM GSSG (black bar) and enzyme activity was determined according to Ref. 36. The PRK activity in untreated control extract was set at 100% (hatched bar). For restoring PRK activity, GSSG-treated extract was desalted in 100 mM Tris-HCl, pH 7.9, using NAP-5 columns and then treated with 10 mM DTT for 15 min (white bar). The data are represented as the mean percentages of maximal activity  $\pm$  S.D. ( $n = 3$ ).

ification by glutathionylation. To analyze the glutathionylation of PGK and RPI *in vitro*, we cloned the corresponding cDNAs and expressed the recombinant proteins in *E. coli*. Both enzymes were purified to homogeneity by nickel affinity chromatography. To confirm their glutathionylation, the reduced enzymes were treated with 2 mM BioGSSG for 1 h, and the presence of glutathione adducts was analyzed by Western blot using anti-biotin antibodies. For both proteins, glutathionylation was detected after BioGSSG treatment, and the signal disappeared in the presence of DTT (Fig. 6). Moreover, no signal was detected when the protein was pretreated with IAM, a specific cysteine alkylating agents, suggesting that the absence of free cysteines prevents biotin labeling via glutathionylation. These results confirm that purified PGK and RPI undergo glutathionylation in the presence of BioGSSG *in vitro*.

A<sub>4</sub>-GAPDH catalyzes the only reductive step of the Calvin-Benson cycle. Glycolytic and photosynthetic GAPDH enzymes contain a strictly conserved catalytic cysteine and are highly sensitive to oxidants (18, 19, 40, 41). Consistently, incubation of *Chlamydomonas* A<sub>4</sub>-GAPDH in the presence of

1 mM  $\text{H}_2\text{O}_2$  led to a complete loss of activity within 2 min (Fig. 7A). This inactivation could not be reversed by DTT treatment (Fig. 7B), suggesting that  $\text{H}_2\text{O}_2$  induced irreversible oxidation, most likely affecting the sulfhydryl group of the active site cysteine. Irreversibly oxidized cysteine thiols are typically converted to sulfinic ( $-\text{SO}_2\text{H}$ ) or sulfonic acids ( $-\text{SO}_3\text{H}$ ) (42). The inactivation of the enzyme was slower in the presence of a 10-fold lower  $\text{H}_2\text{O}_2$  concentration (0.1 mM) (Fig. 7A), but in this case DTT could restore  $\sim 50\%$  of the initial activity (Fig. 7B). This partial recovery, not observed after treatment with 1 mM  $\text{H}_2\text{O}_2$ , indicated that part of the  $\text{A}_4$ -GAPDH molecules were reversibly oxidized, probably to sulfenic acid ( $-\text{SOH}$ ) by primary oxidation of the catalytic cysteine. The involvement of this residue is further supported by the fact that the enzyme substrate, BPGA, which forms a covalent intermediate with the catalytic cysteine, protects  $\text{A}_4$ -GAPDH from  $\text{H}_2\text{O}_2$ -dependent oxidation (Fig. 7A). Moreover, by contrast with the

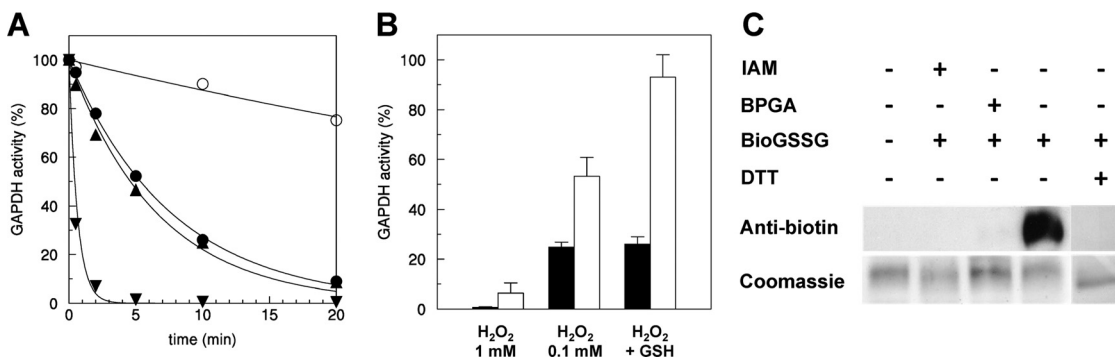
partial recovery after treatment with 0.1 mM  $\text{H}_2\text{O}_2$ , an almost complete recovery of the initial activity after DTT treatment was observed for  $\text{A}_4$ -GAPDH samples treated with 0.1 mM  $\text{H}_2\text{O}_2$  and 0.5 mM GSH (Fig. 7B). This suggested that, in the presence of GSH, the mechanism of inactivation is different and most likely involves glutathionylation. This was confirmed by *in vitro* BioGSSG treatments of purified  $\text{A}_4$ -GAPDH and immunoblotting with anti-biotin antibodies. A strong signal was detected after BioGSSG treatment and was reversed by DTT and blocked by prior alkylation with IAM. Moreover, BPGA also prevented glutathionylation of  $\text{A}_4$ -GAPDH, thereby confirming that the catalytic cysteine is most probably the target of glutathionylation. All of the these data clearly demonstrate that glutathionylation inhibits the activity of *Chlamydomonas*  $\text{A}_4$ -GAPDH but also protects it from irreversible oxidative inactivation. These results are consistent with those reported for *Arabidopsis* GAPDH isoforms (18, 19) and suggest that the mechanisms of redox regulation by glutathionylation of this Calvin-Benson cycle enzyme are conserved between green algae and higher plants. Higher plants contain two types of GAPDH: the  $\text{A}_2\text{B}_2$  form, which is TRX-dependent, and the  $\text{A}_4$  form, which is not regulated by TRX but undergoes glutathionylation. By contrast, algae such as *C. reinhardtii* only contain the  $\text{A}_4$ -GAPDH form. Therefore our results confirm that the only chloroplastic GAPDH isoform present in *Chlamydomonas* can also be redox-regulated by glutathionylation.



**FIG. 6. Analysis of recombinant PGK and RPI glutathionylation with BioGSSG.** Recombinant PGK and RPI were incubated for 1 h in the presence of 2 mM BioGSSG with or without prior incubation with 100 mM IAM. The proteins were resolved by nonreducing SDS-PAGE and transferred to nitrocellulose for anti-biotin immunoblotting. The reversibility of the reaction was assessed by treatment with 20 mM DTT for 30 min as indicated. The Coomassie staining of the gel shows equal loading in each lane.

## DISCUSSION

Our proteomic analysis based on the use of BioGSSG has allowed identification of 225 putative glutathionylated proteins and 56 potential sites of modification. This is the most extensive study on protein glutathionylation in a photosynthetic organism and confirms that this modification is likely to con-



**FIG. 7. Analysis of recombinant  $\text{A}_4$ -GAPDH oxidation and glutathionylation.** A, kinetics of inactivation of  $\text{A}_4$ -GAPDH by different oxidants. Reduced  $\text{A}_4$ -GAPDH (2.5  $\mu\text{M}$ ) was incubated with 1 mM  $\text{H}_2\text{O}_2$  ( $\blacktriangledown$ ) or 0.1 mM  $\text{H}_2\text{O}_2$  ( $\blacktriangle$ ) or 0.1 mM  $\text{H}_2\text{O}_2$  plus 0.5 mM GSH ( $\bullet$ ) or 0.1 mM  $\text{H}_2\text{O}_2$  plus 0.5 mM GSH in the presence of BPGA ( $\circ$ ). Aliquots were withdrawn at the indicated times, and the remaining NADPH-dependent activity was determined. Activity is given as the percentage of the initial activity. B, reversal of  $\text{A}_4$ -GAPDH inactivation by DTT. Reduced  $\text{A}_4$ -GAPDH was incubated for 10 min in the presence of different oxidants as indicated (*black bars*). The reversibility of  $\text{A}_4$ -GAPDH inactivation was assessed by incubation in the presence of 20 mM DTT (*white bars*). The data are represented as mean percentage of maximal activity  $\pm$  S.D. ( $n = 3-5$ ). C, immunoblot analysis of  $\text{A}_4$ -GAPDH glutathionylation with BioGSSG. Reduced  $\text{A}_4$ -GAPDH was incubated for 1 h in the presence of 2 mM BioGSSG with or without prior incubation with 100 mM IAM or BPGA. The proteins were resolved by nonreducing SDS-PAGE and transferred to nitrocellulose for Western blotting with anti-biotin antibodies. The reversibility of the reaction was assessed by treatment with 20 mM DTT for 30 min as indicated. The Coomassie staining of the gel shows equal loading in each lane.

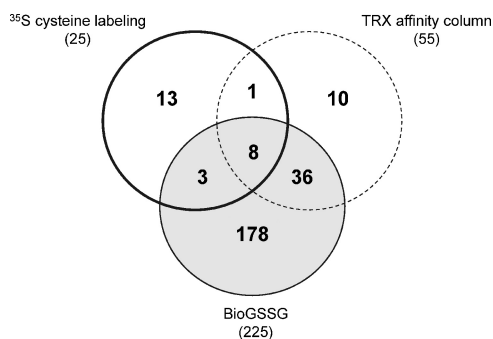


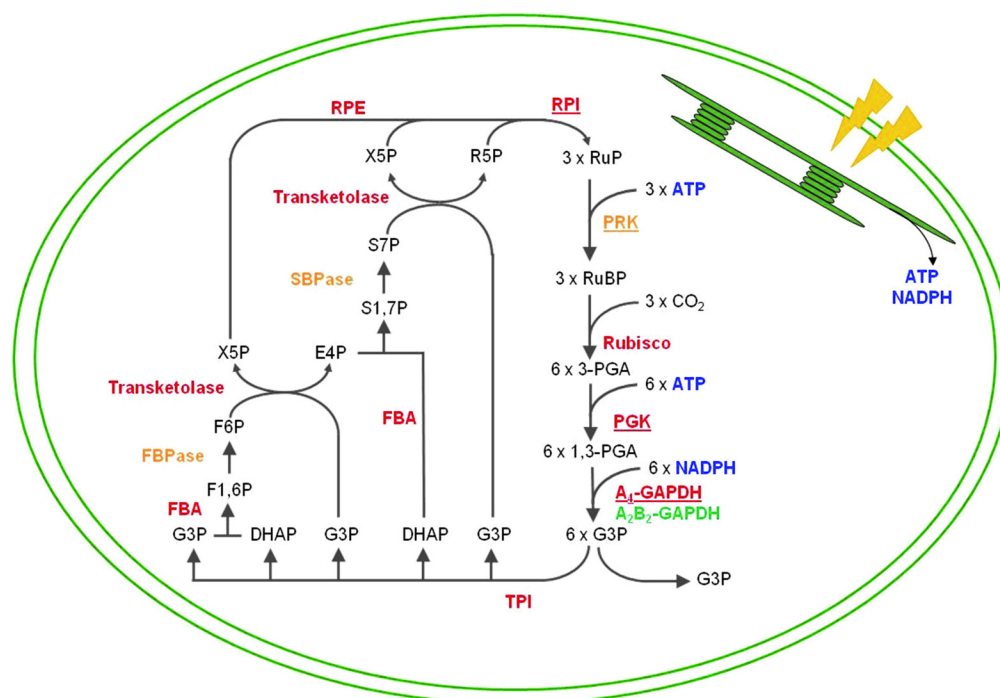
FIG. 8. **Analysis of redox-regulated proteins identified in *Chlamydomonas*.** The Venn diagram shows the distribution of *Chlamydomonas* proteins identified as putative target of S-glutathionylation (BioGSSG), TRX (monocysteinic TRX affinity column (55)), and S-thiolation ( $^{35}\text{S}$ -cysteine radiolabeling (23)).

stitute, as in mammals, an important mechanism of cell regulation and signaling, e.g. under oxidative stress conditions. Our results confirm the utility of BioGSSG for analysis of protein glutathionylation (43, 44). The proteins identified participate in various metabolic pathways and cell processes. Almost one-third of the proteins participate in carbon metabolism including enzymes involved in the Calvin-Benson and TCA cycles, glycolysis/gluconeogenesis, starch, and sucrose metabolism or fatty acid metabolism. These results are consistent with studies in yeast and mammals reporting that numerous glycolytic or TCA cycle enzymes are regulated by glutathionylation (45–50). No protein involved in the regulation of gene expression, such as transcription factors, was identified as glutathionylated in *Chlamydomonas*. This may reflect the low abundance of these enzymes, although this seems unlikely because of the sensitivity of the method employed and the selective enrichment of glutathionylated proteins by streptavidin affinity chromatography. Another possibility is that these enzymes were identified but are found among the 17% of proteins with unknown functions. Indeed, the mechanisms of regulation of gene expression in *Chlamydomonas* are much less known than in organisms where redox-sensitive transcription factors have been identified such as OxyR in *E. coli* (51), Yap1 in yeast (52), or NF- $\kappa$ B or AP-1 in mammals (5, 53, 54). Finally, another possibility could be that the redox-dependent regulation of protein abundance by glutathionylation mainly operates at the translational or post-translational level, as illustrated by the fact that 17% of identified proteins are involved in protein biogenesis and degradation.

Nevertheless, many of the 225 proteins identified were previously found to be redox-regulated in *Chlamydomonas* (Table I and Fig. 8). Among the 25 proteins identified as S-thiolated *in vivo* using [ $^{35}\text{S}$ ]cysteine labeling (23), 11 were found to be glutathionylated with BioGSSG. This suggests that although BioGSSG labeling was performed on *Chlamydomonas* extracts, the proteins and sites of modification identified may be relevant *in vivo*. Some proteins may not be

detected with BioGSSG because this molecule triggers glutathionylation only through thiol-disulfide exchange, whereas other mechanisms may occur *in vivo*. Similarly, 41 among the 55 proteins retained on a TRX affinity column (55) were also identified with BioGSSG. This is consistent with the lack of specificity of monocysteinic TRX columns that can retain proteins harboring different types of oxidative modifications, including protein disulfide bonds and glutathionylation (39). A detailed biochemical characterization of *Chlamydomonas* isocitrate lyase, a protein binding to TRX columns, revealed that the enzyme can be inactivated by glutathionylation of its catalytic cysteine but does not appear to be regulated by TRX (20). Alternatively, it is also possible that some of the identified proteins might be subject to a double regulation by both TRX and glutathionylation. For example, PRK activity is increased by TRXf-dependent reduction of a disulfide bond in the light (56), whereas the enzyme was shown to be inactivated by a glutathionylation treatment in the present study. Similarly, *Chlamydomonas* PRX1 was shown to be reduced by TRX (57) and to undergo glutathionylation *in vitro* (23). For each of these enzymes, further work will be required to confirm the double regulation and to determine whether these mechanisms of redox regulation are independent or interconnected. Finally, 178 of the 225 proteins identified with BioGSSG correspond to new potential targets, not previously suggested to be redox-regulated in *Chlamydomonas*. These results strengthen the potential importance of glutathionylation in photosynthetic organisms for regulation of numerous biological processes and pathways.

The method employed to determine glutathionylation sites appears to be reliable, because many of the sites identified correspond to cysteines known to undergo glutathionylation or oxidative modifications such as Cys<sup>217</sup> in human actin (58), Cys<sup>385</sup> in porcine aconitase (59), Cys<sup>98</sup> in yeast PGK (60), Cys<sup>247</sup> in *Chlamydomonas* isocitrate lyase (20, 23), and Cys<sup>172</sup> in *Chlamydomonas* Rubisco (61). A search for motifs in the primary sequence surrounding the 56 sites identified did not reveal any consensus sequence. Similarly, no motif was identified for S-nitrosylated cysteines (62). Multiple factors appear to contribute to the sensitivity of a given cysteine residue to S-glutathionylation including pK<sub>a</sub>, accessibility, reactivity, and its microenvironment, especially surrounding amino acids (63). A combination of these factors is likely required because protein cysteine residues exhibit a striking differential susceptibility to glutathionylation (9). In the case of human TRX and plant f-type TRXs from diverse species, the glutathionylated cysteine is accessible but also surrounded by a patch of positive charges that could facilitate interaction with the negatively charged glutathione molecule (16, 64). Several other plant TRX isoforms that also contain accessible cysteines but lack surrounding positive charges are apparently not susceptible to glutathionylation (16). Anyway, the identification of glutathionylation sites together with the identity of the modified protein provides important information that



- 1 Protein regulated by f-type TRX (dithiol/disulfide)
- 3 Proteins regulated by f-type TRX (dithiol/disulfide) and likely by glutathionylation
- 10 Proteins likely regulated by glutathionylation
- 4 *Chlamydomonas* proteins confirmed in the present study

FIG. 9. **The Calvin-Benson cycle is a major target of redox regulation.** All of the enzymes of the Calvin-Benson cycle are potentially regulated by TRXf-dependent reduction of a disulfide bond and/or by glutathionylation, as indicated. The Calvin-Benson cycle is represented in a schematic chloroplast. Light energy is used by thylakoids to generate ATP and NADPH consumed by the Calvin-Benson cycle for CO<sub>2</sub> fixation. *Rubisco*, ribulose-1,5-bisphosphate carboxylase/oxygenase; *TPI*, triose phosphate isomerase; *FBA*, fructose-1,6-bisphosphate aldolase; *FBPase*, fructose-1,6-bisphosphatase; *SBPase*, sedoheptulose-1,7-bisphosphatase; *RPE*, ribulose phosphate-3-epimerase; *RuBP*, ribulose-1,5-bisphosphate; 3-PGA, 3-phosphoglycerate; *G3P*, glyceraldehyde-3-phosphate; *DHAP*, dihydroxyacetone phosphate; *F1,6P*, fructose-1,6-bisphosphate; *F6P*, fructose-6-phosphate; *X5P*, xylulose-5-phosphate; *E4P*, erythrose-4-phosphate; *S1,7P*, sedoheptulose-1,7-bisphosphate; *S7P*, sedoheptulose-7-phosphate; *R5P*, ribose-5-phosphate; *RuP*, ribulose-5-phosphate.

will greatly facilitate future studies on the functional role of this modification for the different targets identified.

The Calvin-Benson cycle is regulated by multiple redox post-translational modifications (Fig. 9). Four enzymes of this cycle (fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, A<sub>2</sub>B<sub>2</sub>-GAPDH, and PRK) are well established targets of TRX in chloroplasts. These enzymes have a low activity in the dark and are activated upon illumination by photosynthetically reduced TRX (39, 65). Although chloroplasts contain several isoforms of TRX (f, m, x, y, and z), regulation of Calvin-Benson cycle enzymes is preferentially or exclusively operated by f-type TRXs. Moreover, all Calvin-Benson cycle enzymes from diverse organisms were retained on TRX affinity columns (39, 66–68), suggesting that they might all be redox-regulated. The Calvin-Benson cycle is also regulated either directly or indirectly by glutathionylation. The indirect regulation operates through glutathionylation of TRXf because this modification results in an impaired ability of TRXf to activate its target enzymes in the light (16). Several Calvin-Benson cycle enzymes were reported to be modified directly by glutathionylation including *Arabidopsis* A<sub>4</sub>-GAPDH (19),

fructose-1,6-bisphosphate aldolase, and triose phosphate isomerase (26). Moreover, early studies demonstrated that GSSG could inhibit Calvin cycle enzymes, although at this time GSSG was thought to mediate dark deactivation (69). In the present study, all of the Calvin-Benson cycle enzymes except triose phosphate isomerase were identified as putatively glutathionylated, and four enzymes (PGK, PRK, RPI, and GAPDH) were confirmed. Overall, glutathionylation appears to decrease the activity of Calvin-Benson cycle enzymes. This suggests that under conditions of oxidative stress leading to protein glutathionylation in the chloroplast, the turnover of the Calvin-Benson cycle could be decreased. Such a regulation could have several physiological roles. First, this negative regulation in the presence of ROS could allow a redistribution of reducing power within chloroplasts by decreasing NADPH consumption by the Calvin cycle. This would increase NADPH availability for ROS detoxifying enzymes such as glutathione reductase or monodehydroascorbate reductase. Moreover, this slowing down could also allow a redistribution of electrons to other detoxifying enzymes like peroxiredoxins, glutathione peroxidases, or ascorbate peroxidases with the possi-



## REFERENCES

- ble implication of TRX isoforms whose activity is not apparently regulated by glutathionylation (16, 39). This type of regulation may allow a fine tuning of cell metabolism under stress conditions. The idea that enzymes of the Calvin-Benson cycle could be down-regulated in response to oxidative stress to enhance survival by enabling the plant to redirect energy resources to antioxidant defense was recognized early on in proteomic studies of TRX targets (68). Indeed, carbon dioxide assimilation is among the first chloroplast processes affected when plants experience stress. The Calvin cycle typically slows down with the onset of stress, well before photosynthetic electron flow and ATP synthesis (70, 71). Early results provided evidence that several enzymes of the Calvin cycle such as transketolase or ribulose-3-phosphate-epimerase are sensitive to H<sub>2</sub>O<sub>2</sub> and are sites of H<sub>2</sub>O<sub>2</sub>-dependent inhibition (72). The identification of these enzymes as glutathionylation targets in the present study may provide a link with these initial studies because H<sub>2</sub>O<sub>2</sub> oxidation is an efficient mediator of protein glutathionylation.
- However, current models rather suggest that overreduction of Photosystem I caused by a decreased availability of electron acceptors would tend to increase ROS production. Thus, an alternative hypothesis would be to consider that the slowing down of the Calvin cycle would increase ROS production and thereby reinforce the initial oxidative signal. Clearly, further studies are required to determine the different factors controlling the distribution of reducing equivalents between the different Photosystem I electron acceptors. Finally, all of these data suggest that the Calvin-Benson cycle is tightly regulated by multiple redox post-translational modifications including glutathionylation. The underlying molecular mechanisms will have to be examined thoroughly because they might be crucial for fine tuning of photosynthetic efficiency and carbon fixation under varying environmental conditions.
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- § This article contains [supplemental material](#).
- § These authors contributed equally to this work.
- ¶ Present address: Laboratory of Molecular Plant Physiology, Dept. of Experimental Evolutionary Biology, University of Bologna, Via Irnerio 42, 40126 Bologna, Italy.
- §§ To whom correspondence may be addressed: Institut de Biochimie et Biophysique Moléculaire et Cellulaire, UMR8619 Centre National de la Recherche Scientifique, Université Paris-Sud, Bâtiment 430, 91405 Orsay Cedex, France. E-mail: paulette.decottignies@u-psud.fr.
- ¶¶ To whom correspondence may be addressed: Laboratoire de Biologie Moléculaire et Cellulaire des Eucaryotes, FRE3354 Centre National de la Recherche Scientifique, Université Pierre et Marie Curie, Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France. E-mail: stephane.lemaire@ibpc.fr.
- Choudhary, C., and Mann, M. (2010) Decoding signalling networks by mass spectrometry-based proteomics. *Nat. Rev. Mol. Cell Biol.* **11**, 427–439
  - Foyer, C. H., and Noctor, G. (2009) Redox regulation in photosynthetic organisms: Signaling, acclimation, and practical implications. *Antioxid. Redox Signal.* **11**, 861–905
  - Hancock, J. T. (2009) The role of redox mechanisms in cell signalling. *Mol. Biotechnol.* **43**, 162–166
  - Rouhier, N., Lemaire, S. D., and Jacquot, J. P. (2008) The role of glutathione in photosynthetic organisms: Emerging functions for glutaredoxins and glutathionylation. *Annu. Rev. Plant Biol.* **59**, 143–166
  - Mieyal, J. J., Gallogly, M. M., Qanungo, S., Sabens, E. A., and Shelton, M. D. (2008) Molecular mechanisms and clinical implications of reversible protein S-glutathionylation. *Antioxid. Redox Signal.* **10**, 1941–1988
  - Xiong, Y., Uys, J. D., Tew, K. D., and Townsend, D. M. (2011) S-Glutathionylation: From molecular mechanisms to health outcomes. *Antioxid. Redox Signal.* **15**, 233–270
  - Martinez-Ruiz, A., and Lamas, S. (2007) Signalling by NO-induced protein S-nitrosylation and S-glutathionylation: Convergences and divergences. *Cardiovasc. Res.* **75**, 220–228
  - Chen, C. A., Wang, T. Y., Varadharaj, S., Reyes, L. A., Hemann, C., Talukder, M. A., Chen, Y. R., Druhan, L. J., and Zweier, J. L. (2010) S-Glutathionylation uncouples eNOS and regulates its cellular and vascular function. *Nature* **468**, 1115–1118
  - Dalle-Donne, I., Rossi, R., Colombo, G., Giustarini, D., and Milzani, A. (2009) Protein S-glutathionylation: A regulatory device from bacteria to humans. *Trends Biochem. Sci.* **34**, 85–96
  - Ghezzi, P., and Di Simplicio, P. (2007) Glutathionylation pathways in drug response. *Curr. Opin. Pharmacol.* **7**, 398–403
  - Michelet, L., Zaffagnini, M., Massot, V., Keryer, E., Vanacker, H., Miginiac-Maslow, M., Issakidis-Bourguet, E., and Lemaire, S. D. (2006) Thioredoxins, glutaredoxins, and glutathionylation: New crosstalks to explore. *Photosynth. Res.* **89**, 225–245
  - Shelton, M. D., and Mieyal, J. J. (2008) Regulation by reversible S-glutathionylation: Molecular targets implicated in inflammatory diseases. *Mol. Cells* **25**, 332–346
  - Kehr, S., Jortzik, E., Delahunty, C., Yates, J. R., Rahlfs, S., and Becker, K. (2011) Protein S-glutathionylation in malaria parasites. *Antioxid. Redox Signal.* **15**, 2855–2865
  - Zaffagnini, M., Bedhomme, M., Marchand, C. H., Couturier, J., Gao, X. H., Rouhier, N., Trost, P., and Lemaire, S. D. (2012) Glutaredoxin S12: Unique properties for redox signaling. *Antioxid. Redox Signal.* **16**, 17–32
  - Gelhay, E., Rouhier, N., Gérard, J., Jolivet, Y., Gualberto, J., Navrot, N., Ohlsson, P. I., Wingsle, G., Hirasawa, M., Knaff, D. B., Wang, H., Dizenegrem, P., Meyer, Y., and Jacquot, J. P. (2004) A specific form of thioredoxin h occurs in plant mitochondria and regulates the alternative oxidase. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 14545–14550
  - Michelet, L., Zaffagnini, M., Marchand, C., Collin, V., Decottignies, P., Tsan, P., Lancelin, J. M., Trost, P., Miginiac-Maslow, M., Noctor, G., and Lemaire, S. D. (2005) Glutathionylation of chloroplast thioredoxin f is a redox signaling mechanism in plants. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 16478–16483
  - Dixon, D. P., Fordham-Skelton, A. P., and Edwards, R. (2005) Redox regulation of a soybean tyrosine-specific protein phosphatase. *Biochemistry* **44**, 7696–7703
  - Holtgreve, S., Gohlke, J., Starmann, J., Druce, S., Klocke, S., Altmann, B., Wojtera, J., Lindermayr, C., and Scheibe, R. (2008) Regulation of plant cytosolic glyceraldehyde 3-phosphate dehydrogenase isoforms by thiol modifications. *Physiol. Plant* **133**, 211–228
  - Zaffagnini, M., Michelet, L., Marchand, C., Sparla, F., Decottignies, P., Le Maréchal, P., Miginiac-Maslow, M., Noctor, G., Trost, P., and Lemaire, S. D. (2007) The thioredoxin-independent isoform of chloroplastic glyceraldehyde-3-phosphate dehydrogenase is selectively regulated by glutathionylation. *FEBS J.* **274**, 212–226
  - Bedhomme, M., Zaffagnini, M., Marchand, C. H., Gao, X. H., Moslonka-Lefebvre, M., Michelet, L., Decottignies, P., and Lemaire, S. D. (2009) Regulation by glutathionylation of isocitrate lyase from *Chlamydomonas reinhardtii*. *J. Biol. Chem.* **284**, 36282–36291
  - Leferink, N. G., van Duijn, E., Barendregt, A., Heck, A. J., and van Berkel, W. J. (2009) Galactonolactone dehydrogenase requires a redox-sensitive thiol for optimal production of vitamin C. *Plant Physiol.* **150**, 596–605

22. Palmieri, M. C., Lindermayr, C., Bauwe, H., Steinhauser, C., and Durner, J. (2010) Regulation of plant glycine decarboxylase by S-nitrosylation and glutathionylation. *Plant Physiol.* **152**, 1514–1528
23. Michelet, L., Zaffagnini, M., Vanacker, H., Le Maréchal, P., Marchand, C., Schroda, M., Lemaire, S. D., and Decottignies, P. (2008) *In vivo* targets of S-thiolation in *Chlamydomonas reinhardtii*. *J. Biol. Chem.* **283**, 21571–21578
24. Noguera-Mazon, V., Lemoine, J., Walker, O., Rouhier, N., Salvador, A., Jacquot, J. P., Lancelin, J. M., and Krimm, I. (2006) Glutathionylation induces the dissociation of 1-Cys D-peroxiredoxin non-covalent homodimer. *J. Biol. Chem.* **281**, 31736–31742
25. Tarrago, L., Laugier, E., Zaffagnini, M., Marchand, C., Le Maréchal, P., Rouhier, N., Lemaire, S. D., and Rey, P. (2009) Regeneration mechanisms of *Arabidopsis thaliana* methionine sulfoxide reductases B by glutaredoxins and thioredoxins. *J. Biol. Chem.* **284**, 18963–18971
26. Ito, H., Iwabuchi, M., and Ogawa, K. (2003) The sugar-metabolic enzymes aldolase and triose-phosphate isomerase are targets of glutathionylation in *Arabidopsis thaliana*: Detection using biotinylated glutathione. *Plant Cell Physiol.* **44**, 655–660
27. Dixon, D. P., Skipsey, M., Grundy, N. M., and Edwards, R. (2005) Stress-induced protein S-glutathionylation in *Arabidopsis*. *Plant Physiol.* **138**, 2233–2244
28. Rochaix, J. D., Mayfield, S. P., Goldschmidt-Clermont, M., and Erickson, J. (1988) *Plant molecular biology: A practical approach*, IRL, Oxford
29. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
30. Ellman, G. L. (1959) Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82**, 70–77
31. Gillet, S., Decottignies, P., Chardonnet, S., and Le Maréchal, P. (2006) Cadmium response and redoxin targets in *Chlamydomonas reinhardtii*: A proteomic approach. *Photosynth. Res.* **89**, 201–211
32. Pasternak, T. P., Prinsen, E., Ayaydin, F., Miskolczi, P., Potters, G., Asard, H., Van Onckelen, H. A., Dudits, D., and Fehér, A. (2002) The role of auxin, pH, and stress in the activation of embryogenic cell division in leaf protoplast-derived cells of alfalfa. *Plant Physiol.* **129**, 1807–1819
33. Schwartz, D., and Gygi, S. P. (2005) An iterative statistical approach to the identification of protein phosphorylation motifs from large-scale data sets. *Nat. Biotechnol.* **23**, 1391–1398
34. Petersen, B., Petersen, T. N., Andersen, P., Nielsen, M., and Lundegaard, C. (2009) A generic method for assignment of reliability scores applied to solvent accessibility predictions. *BMC Struct. Biol.* **9**, 51
35. Graciet, E., Lebreton, S., Camadro, J. M., and Gontero, B. (2003) Characterization of native and recombinant A4 glyceraldehyde 3-phosphate dehydrogenase: Kinetic evidence for conformational changes upon association with the small protein CP12. *Eur. J. Biochem.* **270**, 129–136
36. Gontero, B., Cárdenas, M. L., and Ricard, J. (1988) A functional five-enzyme complex of chloroplasts involved in the Calvin cycle. *Eur. J. Biochem.* **173**, 437–443
37. Chapman-Smith, A., and Cronan, J. E., Jr. (1999) The enzymatic biotinylation of proteins: a post-translational modification of exceptional specificity. *Trends Biochem. Sci.* **24**, 359–363
38. Healy, S., McDonald, M. K., Wu, X., Yue, W. W., Kochan, G., Oppermann, U., and Gravel, R. A. (2010) Structural impact of human and *Escherichia coli* biotin carboxyl carrier proteins on biotin attachment. *Biochemistry* **49**, 4687–4694
39. Lemaire, S. D., Michelet, L., Zaffagnini, M., Massot, V., and Issakidis-Bourguet, E. (2007) Thioredoxins in chloroplasts. *Curr. Genet.* **51**, 343–365
40. Grant, C. M., Quinn, K. A., and Dawes, I. W. (1999) Differential protein S-thiolation of glyceraldehyde-3-phosphate dehydrogenase isoenzymes influences sensitivity to oxidative stress. *Mol. Cell. Biol.* **19**, 2650–2656
41. Talfournier, F., Colloc'h, N., Mornon, J. P., and Branlant, G. (1998) Comparative study of the catalytic domain of phosphorylating glyceraldehyde-3-phosphate dehydrogenases from bacteria and archaea via essential cysteine probes and site-directed mutagenesis. *Eur. J. Biochem.* **252**, 447–457
42. Poole, L. B., Karplus, P. A., and Claiborne, A. (2004) Protein sulfenic acids in redox signaling. *Annu. Rev. Pharmacol. Toxicol.* **44**, 325–347
43. Brennan, J. P., Miller, J. I., Fuller, W., Wait, R., Begum, S., Dunn, M. J., and Eaton, P. (2006) The utility of *N,N*-biotinyl glutathione disulfide in the study of protein S-glutathionylation. *Mol. Cell. Proteomics* **5**, 215–225
44. Lind, C., Gerdes, R., Hamnell, Y., Schuppe-Koistinen, I., von Löwenhielm, H. B., Holmgren, A., and Cotgreave, I. A. (2002) Identification of S-glutathionylated cellular proteins during oxidative stress and constitutive metabolism by affinity purification and proteomic analysis. *Arch. Biochem. Biophys.* **406**, 229–240
45. Applegate, M. A., Humphries, K. M., and Szveda, L. I. (2008) Reversible inhibition of  $\alpha$ -ketoglutarate dehydrogenase by hydrogen peroxide: Glutathionylation and protection of lipoic acid. *Biochemistry* **47**, 473–478
46. Fratelli, M., Demol, H., Puype, M., Casagrande, S., Villa, P., Eberini, I., Vandekerckhove, J., Gianazza, E., and Ghezzi, P. (2003) Identification of proteins undergoing glutathionylation in oxidatively stressed hepatocytes and hepatoma cells. *Proteomics* **3**, 1154–1161
47. Kil, I. S., and Park, J. W. (2005) Regulation of mitochondrial NADP<sup>+</sup>-dependent isocitrate dehydrogenase activity by glutathionylation. *J. Biol. Chem.* **280**, 10846–10854
48. Mohr, S., Hallak, H., de Boitte, A., Lapetina, E. G., and Brüne, B. (1999) Nitric oxide-induced S-glutathionylation and inactivation of glyceraldehyde-3-phosphate dehydrogenase. *J. Biol. Chem.* **274**, 9427–9430
49. Shenton, D., and Grant, C. M. (2003) Protein S-thiolation targets glycolysis and protein synthesis in response to oxidative stress in the yeast *Saccharomyces cerevisiae*. *Biochem. J.* **374**, 513–519
50. Shin, S. W., Oh, C. J., Kil, I. S., and Park, J. W. (2009) Glutathionylation regulates cytosolic NADP<sup>+</sup>-dependent isocitrate dehydrogenase activity. *Free Radic. Res.* **43**, 409–416
51. Kim, S. O., Merchant, K., Nudelman, R., Beyer, W. F., Jr., Keng, T., DeAngelo, J., Hausladen, A., and Schuster, J. S. (2002) OxyR: A molecular code for redox-related signaling. *Cell* **109**, 383–396
52. Toledano, M. B., Delaunay, A., Monceau, L., and Tacnet, F. (2004) Microbial H<sub>2</sub>O<sub>2</sub> sensors as archetypical redox signaling modules. *Trends Biochem. Sci.* **29**, 351–357
53. Pineda-Molina, E., Klatt, P., Vázquez, J., Marina, A., García de Lacoba, M., Pérez-Sala, D., and Lamas, S. (2001) Glutathionylation of the p50 subunit of NF- $\kappa$ B: A mechanism for redox-induced inhibition of DNA binding. *Biochemistry* **40**, 14134–14142
54. Reynaert, N. L., van der Vliet, A., Guala, A. S., McGovern, T., Hristova, M., Pantano, C., Heintz, N. H., Heim, J., Ho, Y. S., Matthews, D. E., Wouters, E. F., and Janssen-Heininger, Y. M. (2006) Dynamic redox control of NF- $\kappa$ B through glutaredoxin-regulated S-glutathionylation of inhibitory  $\kappa$ B kinase  $\beta$ . *Proc. Natl. Acad. Sci. U.S.A.* **103**, 13086–13091
55. Lemaire, S. D., Guillon, B., Le Maréchal, P., Keryer, E., Miginiac-Maslow, M., and Decottignies, P. (2004) New thioredoxin targets in the unicellular photosynthetic eukaryote *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 7475–7480
56. Marri, L., Zaffagnini, M., Collin, V., Issakidis-Bourguet, E., Lemaire, S. D., Pupillo, P., Sparla, F., Miginiac-Maslow, M., and Trost, P. (2009) Prompt and easy activation by specific thioredoxins of calvin cycle enzymes of *Arabidopsis thaliana* associated in the GAPDH/CP12/PRK supramolecular complex. *Mol. Plant* **2**, 259–269
57. Goyer, A., Haslekås, C., Miginiac-Maslow, M., Klein, U., Le Marechal, P., Jacquot, J. P., and Decottignies, P. (2002) Isolation and characterization of a thioredoxin-dependent peroxidase from *Chlamydomonas reinhardtii*. *Eur. J. Biochem.* **269**, 272–282
58. Hamnell-Pamment, Y., Lind, C., Palmberg, C., Bergman, T., and Cotgreave, I. A. (2005) Determination of site-specificity of S-glutathionylated cellular proteins. *Biochem. Biophys. Res. Commun.* **332**, 362–369
59. Han, D., Canali, R., Garcia, J., Aguilera, R., Gallaher, T. K., and Cadenas, E. (2005) Sites and mechanisms of aconitase inactivation by peroxynitrite: Modulation by citrate and glutathione. *Biochemistry* **44**, 11986–11996
60. McDonagh, B., Ogueta, S., Lasarte, G., Padilla, C. A., and Bárcena, J. A. (2009) Shotgun redox proteomics identifies specifically modified cysteines in key metabolic enzymes under oxidative stress in *Saccharomyces cerevisiae*. *J. Proteomics* **72**, 677–689
61. Moreno, J., and Spreitzer, R. J. (1999) C172S substitution in the chloroplast-encoded large subunit affects stability and stress-induced turnover of ribulose-1,5-bisphosphate carboxylase/oxygenase. *J. Biol. Chem.* **274**, 26789–26793
62. Marino, S. M., and Gladyshev, V. N. (2010) Structural analysis of cysteine S-nitrosylation: A modified acid-based motif and the emerging role of trans-nitrosylation. *J. Mol. Biol.* **395**, 844–859
63. Dalle-Donne, I., Milzani, A., Gagliano, N., Colombo, R., Giustarini, D., and

- Rossi, R. (2008) Molecular mechanisms and potential clinical significance of S-glutathionylation. *Antioxid. Redox Signal.* **10**, 445–473
64. Casagrande, S., Bonetto, V., Fratelli, M., Gianazza, E., Eberini, I., Massignan, T., Salmona, M., Chang, G., Holmgren, A., and Ghezzi, P. (2002) Glutathionylation of human thioredoxin: A possible crosstalk between the glutathione and thioredoxin systems. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 9745–9749
65. Schürmann, P., and Buchanan, B. B. (2008) The ferredoxin/thioredoxin system of oxygenic photosynthesis. *Antioxid. Redox Signal.* **10**, 1235–1274
66. Lindahl, M., Mata-Cabana, A., and Kieselbach, T. (2011) The disulfide proteome and other reactive cysteine proteomes: analysis and functional significance. *Antioxid. Redox Signal.* **14**, 2581–2642
67. Montrichard, F., Alkhaloui, F., Yano, H., Vensel, W. H., Hurkman, W. J., and Buchanan, B. B. (2009) Thioredoxin targets in plants: The first 30 years. *J. Proteomics* **72**, 452–474
68. Balmer, Y., Koller, A., del Val, G., Manieri, W., Schürmann, P., and Buchanan, B. B. (2003) Proteomics gives insight into the regulatory function of chloroplast thioredoxins. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 370–375
69. Wolosiuk, R. A., and Buchanan, B. B. (1977) Thioredoxin and glutathione regulate photosynthesis in chloroplasts. *Nature* **266**, 565–567
70. Lawlor, D. W., and Cornic, G. (2002) Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. *Plant Cell Environ.* **25**, 275–294
71. Weis, E. (1981) Reversible heat-inactivation of the calvin cycle: A possible mechanism of the temperature regulation of photosynthesis. *Planta* **151**, 33–39
72. Kaiser, W. (1976) The effect of hydrogen peroxide on CO<sub>2</sub> fixation of isolated intact chloroplasts. *Biochim. Biophys. Acta* **440**, 476–482