Glutathionylation in the Photosynthetic Model Organism Chlamydomonas reinhardtii: A Proteomic Survey*§

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

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 ‡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 †Laboratoire de Biochimie et Biophysique Moléculaire et Cellulaire, UMR8619 Centre National de la Recherche Scientifique, Université Paris-Sud, 91405 Orsay Cedex, France, the **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, BP 71, 13402 Marseille Cedex 20, France, and the §§ Laboratoire de Biologie Intégrative, URA296 Centre National de la Recherche Scientifique, Commissariat à l’Énergie 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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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 (γ-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 [35S]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-γ-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, [35S]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 WVR 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 μE·m−2·s−1). Two liters of Chlamydomonas culture at 5–7 × 106 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 μM 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 μl, 64 mM) was added to GSSG (50 μl, 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 μl of 0.6 M ammonium bicarbonate (NH₄HCO₃).

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 γ-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 γ-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 μl, 25 mM) was added to glutathione ethyl ester (300 μl, 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 μl 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 M⁻¹ 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 6–8 × 10⁶ cells/ml. After centrifugation (4,000 g, 3 min), the pellet was washed once with fresh TAP medium and resuspended to 8 × 10⁶ 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 H₂O₂ 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 Protein system. Sixteen μl 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 μl. 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 100 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 2 ml of washing buffer without NaCl. Finally, bound proteins were eluted with 20 mM DTT in 10 mM ammonium bicarbonate (NH₄HCO₃). 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 μl of 3% acetonitrile (CH₃CN) containing 0.1% v/v formic acid and analyzed by mass spectrometry.

**Tandem Mass Spectrometry**—Nanoflow LC-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 μl) were loaded at a flow of 4 μl/min onto the enrichment column equilibrated in 3% CH₃CN 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% CH₃CN in 0.1% formic acid over 3 min and 20–50% CH₃CN in 0.1% formic acid over 12 min) followed by an isocratic step at 70% CH₃CN in 0.1% formic acid for 5 min and an equilibration step with 3% CH₃CN 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 l/min. Helium was used as cooling and fragmentation gas at a pressure of 6 × 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 μg 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 μl 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 Icons Search and Phenyx.

For identifications with Mascot, the NCBI Ref 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 Fast-a 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 follows: peptide length ≥
Glutathionylation Targets in Chlamydomonas

Glutathionylation was analyzed in Chlamydomonas gluta-
mation 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.

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.

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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 specific reaction of BioGSSG with noncysteine amino acids. On
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 ε-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 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 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, tubulin α, 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
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 into subcategories, thereby revealing that the process containing the highest number of putative targets of glutathionylation is photosynthesis and especially CO$_2$ 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 Chlamydomonas 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. Glutathionylated 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 1

**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 [35S]cysteine labeling (23). Column T shows proteins identified as thioredoxin targets in C. reinhardtii (55).

| Protein name | RefSeq | # | S | Glutathionylated cysteine-containing peptide |
|--------------|--------|---|---|---------------------------------------------|
| Apoferritin 1 | XP_001692808 | × | × | K.TPSGRTIIEG5DPYTVLDAEREACLGLDLPSG<sup>5</sup>R.A |
| Fructose-1,6-bisphosphatase | XP_001690872 | × | × | K.GTVDKLAVSVNLTS<sup>2</sup>K.Q |
| Phosphoglycerate kinase | XP_001699523 | × | × | K.OXYGTVH5PGQGLAAGMK.I |
| Phosphoribokinase | XP_001694038 | × | × | K.RC<sup>511</sup>TIQGSSDAVAEQAGMAK.E |
| Ribulose bisphosphate carboxylase large chain | NP_958405 | × | × | K.VDC<sup>515</sup>ICGDSVEK.A |
| Transketolase | XP_001701881 | × | × | K.SGPQYMG<sup>44</sup>APKTVLSEVNEK.V |
| Enolase | XP_001702971 | × | × | K.KVEENLIGG5VRK.E |
| Fructose-1,6-bisphosphate aldolase | XP_001700459 | × | × | K.LTENIC<sup>317</sup>TQVRDGLTNPVR.V |
| Phosphoenolpyruvate carboxykinase, splice variant | XP_001694964 | × | × | K.RABFYG<sup>57</sup>VR.G |
| Glyoxylate cycle | | | | |
| Isocitrate lyase | XP_001695331 | × | × | K.HAPVPAOLDR.R |
| Malate synthase | XP_001695632 | × | × | K.QETLQG<sup>39</sup>TNSYEDMA.R |
| TCA cycle | | | | |
| Acetate hydratase, mitochondrial | XP_001697902 | × | × | K.RGTVQG<sup>54</sup>TQTVRQWMWVIR.Y |
| Starch and sucrose metabolism | | | | |
| ADP-glucose pyrophosphorylase large subunit | XP_001693447 | × | × | K.LIDYPMS<sup>112</sup>INGRIS.K |
| Phosphorylase, plastidial | | | | |
| Metabolism of thiamine, chlorophyll, and terpenoids | | | | |
| Full-length thiazole biosynthetic enzyme | XP_001698672 | × | × | K.INDQFQAOIQTVLGASACAGL<sup>185</sup>AFELOR.I |
| Magnesium-chelatase subunit chloroplast | XP_001691232 | × | × | K.KIMPGLPLGATD<sup>185</sup>QTIDIER.A |
| Amino acids and nitrogen metabolism (3 proteins) | | | | |
| Acetohydroxy acid isomeroreductase | XP_001702649 | × | × | K.GHPPSR<sup>171</sup>NESIEAVSDLNPYMLMAR.G |
| Adenosylhomocysteinase | XP_001693399 | × | × | K.DIAEADVLEGLAESPMG<sup>83</sup>SR.S |
| Protein biogenesis and degradation (11 proteins) | | | | |
| Protein biosynthesis, translation | | | | |
| Eukaryotic translation elongation factor 1α | XP_001696658 | × | × | K.EHLSVT<sup>46</sup>CHVDSGK.S |
| Elongation factor 1α | XP_001703215 | × | × | K.LC<sup>58</sup>EVLYQVETQGQAGQLQGTYNLTR.R |
| Elongation factor Tu | XP_001696344 | × | × | K.RHYAOQ<sup>39</sup>PHYAZVY.N |
| Ribosomal protein L4 | XP_001694804 | × | × | K.VQCC<sup>53</sup>CHVDSGK.V |
| Ribosomal protein L8 | XP_001700532 | × | × | K.RQQAPG<sup>84</sup>NRI.R |
| Ribosomal protein L21 | XP_001698624 | × | × | K.TAA<sup>146</sup>SREELFTIR.R |
| Ribosomal protein S5 | XP_001695400 | × | × | K.TIAE<sup>144</sup>LABLWNAK.K |
| Ribosomal protein S6 | XP_001695102 | × | × | K.CG<sup>143</sup>MPOLAVLHNVU.V.K |
| Ribosomal protein S12 | XP_001698669 | × | × | K.KVQ<sup>115</sup>AVITDGSSEATGSLQLEGK.S |
| Folding/molecular chaperones | | | | |
| Chaperonin 60B2 | XP_001692504 | × | × | K.RC<sup>337</sup>SLEHADVSEK.T |
| Degradation | | | | |
| E3 ubiquitin ligase | XP_001690964 | × | × | K.GLLDLT<sup>111</sup>QTVAQMQ.K.G |
| Energy/ATP metabolism (1 protein) | | | | |
| Vacular ATP synthase subunit E | XP_001692936 | × | × | K.RHE<sup>106</sup>SNLDR.L |

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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 [35S]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 A4-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 modification 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 agent, 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.

A4-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 A4-GAPDH in the presence of...
1 mM H$_2$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 H$_2$O$_2$ induced irreversible oxidation, most likely affecting the sulfhydryl group of the active site cysteine. Irreversibly oxidized cysteine thiol is typically converted to sulfenic (-SO$_2$H) or sulfonic acids (-SO$_3$H) (42). The inactivation of the enzyme was slower in the presence of a 10-fold lower H$_2$O$_2$ concentration (0.1 mM) (Fig. 7A), but in this case DTT could restore ~50% of the initial activity (Fig. 7B). This partial recovery, not observed after treatment with 1 mM H$_2$O$_2$, indicated that part of the A$_4$-GAPDH molecules were reversibly oxidized, probably to sulfenic acid (-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 A$_4$-GAPDH from H$_2$O$_2$-dependent oxidation (Fig. 7A). Moreover, by contrast with the partial recovery after treatment with 0.1 mM H$_2$O$_2$, an almost complete recovery of the initial activity after DTT treatment was observed for A$_4$-GAPDH samples treated with 0.1 mM H$_2$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 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 A$_4$-GAPDH, thereby confirming that the catalytic cysteine is most probably the target of glutathionylation. All of these data clearly demonstrate that glutathionylation inhibits the activity of Chlamydomonas 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 A$_2$B$_2$ form, which is TRX-dependent, and the A$_4$ form, which is not regulated by TRX but undergoes glutathionylation. By contrast, algae such as C. reinhardtii only contain the 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.

**FIG. 7.** Analysis of recombinant A$_4$-GAPDH oxidation and glutathionylation. A, kinetics of inactivation of A$_4$-GAPDH by different oxidants. Reduced A$_4$-GAPDH (2.5 μM) was incubated with 1 mM H$_2$O$_2$ (white bars) or 0.1 mM H$_2$O$_2$ (black bars) or 0.1 mM H$_2$O$_2$ plus 0.5 mM GSH (black bars) or 0.1 mM H$_2$O$_2$ plus 0.5 mM GSH in the presence of BPGA (black bars). 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 A$_4$-GAPDH inactivation by DTT. Reduced A$_4$-GAPDH was incubated for 10 min in the presence of different oxidants as indicated (black bars). The reversibility of 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 ± S.D. (n = 3–5). C, immunoblot analysis of A$_4$-GAPDH glutathionylation with BioGSSG. Reduced 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.

**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-
Glutathionylation Targets in Chlamydomonas

**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 (35S-cysteine radiolabeling (23)).

Glutathionylation targets include numerous glycolytic or TCA cycle enzymes that are regulated by redox-dependent mechanisms (56). 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). A combination of these factors is likely required because protein cysteine residues exhibit a striking sensitivity, reactivity, and its microenvironment, especially surrounding amino acids (63). A combination of these factors is likely required because protein cysteine residues exhibit a striking sensitivity, reactivity, and its microenvironment, especially surrounding amino acids (63). 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 Cys217 in human actin (58), Cys385 in porcine aconitase (59), Cys247 in yeast PGK (60), and Cys247 in Chlamydomonas isocitrate lyase (20, 23), and Cys172 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 pKₐ, 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 glutathionylation targets may also 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 TRX-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.

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 [35S]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 TRX-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.

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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, A2B2-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 A2-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-
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 H₂O₂ and are sites of H₂O₂-dependent inhibition (72). The identification of these enzymes as glutathionylation targets in the present study may provide a link with these initial studies because H₂O₂ 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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**§§ 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: paullete.decottignies@u-psud.fr.

¶¶ To whom correspondence may be addressed: Laboratoire de Biologie Moléculaire et Cellulaire des Eucaryotes, FREN354 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@ibpca.fr.

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