Glutathionylation is the major form of S-thiolation in cells. This reversible redox post-translational modification consists of the formation of a mixed disulfide between a free thiol on a protein and a molecule of glutathione. This recently described modification, which is considered to occur under oxidative stress, can protect cysteine residues from irreversible oxidation, and alter positively or negatively the activity of diverse proteins. This modification and its targets have been mainly studied in non-photosynthetic organisms so far. We report here the first proteomic approach performed in vivo on photosynthetically competent cells, using the eukaryotic unicellular green alga *Chlamydomonas reinhardtii* with radiolabeled [35S]cysteine to label the glutathione pool and diamide as oxidant. This method allowed the identification of 25 targets, mainly chloroplastic, involved in various metabolic processes. Several targets are related to photosynthesis, such as the Calvin cycle enzymes phosphoglycerate kinase and ribose-5-phosphate isomerase. A number of targets, such as chaperones and peroxiredoxins, are related to stress responses. The glutathionylation of HSP70B, chloroplastic 2-Cys peroxiredoxin and isocitrate lyase was confirmed in vitro on purified proteins and the targeted residues were identified.

Glutathione is a tripeptide (γ-l-glutamyl-l-cysteinylglycine) of 307 Da found in almost all living organisms. Glutathione synthesis occurs in two steps and requires two enzymes: γ-l-glutamyl-l-cysteine synthetase and glutathione synthetase. It is the most abundant free soluble thiol in cells, widely considered to constitute a redox buffer. Indeed, it exists in two different forms: the reduced form (GSH), which is the major form, and the oxidized form (GSSG). Glutathione is involved in various cellular processes in photosynthetic organisms, including detoxification of reactive oxygen species, xenobiotics and heavy metals, G1/S cell cycle transition, cell differentiation, flowering, anthocyanin accumulation, and programmed cell death, but also resistance toward pathogens (reviewed in Refs. 1–3).

Glutathione is also involved in a reversible post-translational modification, named glutathionylation, consisting of the formation of a mixed disulfide between an accessible free thiol on a protein and a molecule of glutathione. The one or more exact mechanisms leading to protein glutathionylation remain to be determined, but this modification seems to occur under oxidative stress conditions. Protein glutathionylation can protect cysteine residues from irreversible oxidation but can also regulate either positively or negatively the activity of many proteins (reviewed in Refs. 4–6).

To date, this modification has been mainly studied in non-photosynthetic organisms where proteomic studies allowed identification of ~150 targets, whereas very little is known in photosynthetic organisms. Indeed, the first proteins reported to undergo glutathionylation in plants were two glutathione S-transferases (7). Glutathionylation of two thioredoxins (8, 9), a protein tyrosine phosphatase (10), and A1-glyceraldehyde-3-phosphate dehydrogenase (A1-GAPDH) (11) have been reported. Glutathionylation targets have also been identified in *Arabidopsis* cell cultures using biotinylated glutathione (12, 13). Due to technical difficulties, only 11 targets were identified in vivo. Using the same approach, 72 putative targets were identified in vitro, but only a few were chloroplastic, probably due to the use of dark-grown cells (12). However, considering that in photosynthetic organisms chloroplasts are the main sites of reactive oxygen species production in the light, regulation by glutathionylation could play an important role in this cellular compartment. To investigate glutathionylation in vivo, we adapted to *Chlamydomonas* the methods developed for identification of S-thiolated proteins in human cells (14, 15). This method is based on the use of [15S]cysteine to label the glutathione pool in light-grown cultures of *Chlamydomonas reinhardtii*, a photosynthetic unicellular eukaryote, while protein translation is inhibited. After performing an oxidative stress treatment, radiolabeled proteins were identified by mass spectrometry. We report here the first large scale study of S-thiolation targets on photosynthetically competent cells. This method allowed the identification of 25 targets, and glutathionylation was confirmed in vitro for three of them.

**EXPERIMENTAL PROCEDURES**

Reagents—Unless otherwise specified, all chemicals were obtained from Sigma-Aldrich.
**S-Thiolation Targets in Chlamydomonas**

*Chlamydomonas* Cultures—A cw15 cell wall-less strain of *Chlamydomonas* was grown in Tris acetate phosphate medium (TAP) prepared as described previously (16), with up to 5 × 10⁶ cells/ml under illumination at 100 μE.m⁻².s⁻¹ and at 25 °C.

**Glutathione Measurements**—Total and oxidized glutathione were measured as described previously (17) starting from a minimum of 5 × 10⁶ cells obtained by centrifugation (3000 × g, 5 min, 4 °C). Cells were broken in acid extraction buffer using an automatic grinder (MM 300, Retsch).

**Labeling of Thiolated Proteins and Protein Extraction**—Protein labeling was performed at 25 °C, under constant stirring in 1 liter of a *Chlamydomonas* culture at 5 × 10⁶ cells/ml. Protein translation was inhibited by the addition of 10 μg/ml (w/v) cycloheximide and 200 μg/ml (w/v) chloramphenicol in the growth medium. After a 15-min incubation, cells were pelleted and resuspended in a 1/100 volume of TAP medium, 20 μg/ml (w/v) cycloheximide, and 400 μg/ml (w/v) chloramphenicol (TAP plus inhibitors medium). After an additional 15-min incubation with the two inhibitors, cells were incubated with 1 mM [³⁵S]cysteine (50 μCi/ml, Amersham Biosciences) for 3 h. Cells were harvested by centrifugation and resuspended in the same volume of TAP plus inhibitors medium before incubation with 1 mM diamide for 15 min. Cells were washed once with the TAP plus inhibitors medium and resuspended in the same volume of 30 mM Tris-HCl, pH 7.9, supplemented with 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 purification was determined by the Bradford assay (Bio-Rad).

**Trichloroacetic Acid Precipitation and Two-dimensional Gel Electrophoresis**—Proteins were precipitated by 10% trichloroacetic acid (final concentration), centrifuged, and successively washed with 10% trichloroacetic acid (three times), 5 mM NH₄HCO₃, and distilled water. The pellets were vacuum-dried and resolubilized in 600 μl of urea buffer (8 M urea, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.2% ampholytes, 0.001% (w/v) bromphenol blue). Isoelectric focusing was performed on dry immobilized pH gradient strips (pH range, non-linear 3–10 or linear 4–7, 17-cm length) as recommended by the manufacturer (Bio-Rad), in the absence of reducing agents. In the second dimension, proteins were separated in 12% polyacrylamide gels with a Protean II XL system (Bio-Rad) and detected by staining with Coomassie Brilliant Blue R-250, with no reducing treatment. De-glutathionylation treatments were performed on the polyacrylamide gels and consisted of successive baths at room temperature in 40% (v/v) ethanol, 10% (v/v) acetic acid (16 h); water (2 × 10 min); 30 mM Tris-HCl, pH 7.9 (10 min); 30 mM Tris-HCl, pH 7.9, 1 mM DTT (3 × 40 min), when necessary; and water (3 × 10 min) prior to protein staining. Radioactivity was analyzed by fluorography with a PhosphorImager (Amersham Biosciences) after drying.

**Protein Identification**—Spots were excised and destained, and proteins were digested with trypsin. Peptides were extracted as described (18) and submitted to matrix-assisted laser desorption ionization time-of-flight analysis on a Voyager DE-STR mass spectrometer (PerSeptive Biosystems, Framingham, MA). Proteins were identified using the search program Mascot reducing the NCBI data base to the *Viridiplantae*. Two missed cleavage sites, oxidation of methionine, modification of cysteines by iodoacetamide, and a 50-ppm tolerance in mass values were considered in searches.

**Plasmid Construction for Expression of CrICL in Escherichia coli**—The cDNA-encoding *C. reinhardtii* isocitrate lyase (CrICL) was obtained by PCR on the expressed sequence tag clone AV622994 obtained from the Kazusa DNA Research Institute (Chiba, Japan), using a forward primer introducing an Ndel restriction site (underlined) at the start codon (bolded): 5’-CGTCCTCATATGGCCCTCAACGGCGCTG-3’; and a reverse primer introducing a BamHI restriction site (underlined) downstream of the stop codon: 5’-GCCTGGATCCT-CAGTGGTGGAACCTGCTCT-3’. CrICL was cloned in a modified pET-3c vector containing additional codons upstream of the Ndel site to express a His-tagged protein with seven N-terminal histidines. The sequence was checked by sequencing.

**Protein Purification**—Recombinant CrICL was produced using the pET-3c-HIS/BL21 expression system. Bacteria were grown in LB medium at 37 °C, and the production was induced with 200 μM isopropyl-β-D-thiogalactopyranoside at 27 °C. Cells were harvested by centrifugation, resuspended in 50 mM HEPES-NaOH buffer, pH 7.2, broken using a French press (6.9 × 10⁷ Pa), and cell debris was removed by centrifugation. The supernatant was applied onto a Ni²⁺ HiTrap chelating resin (HiSelect® nickel affinity gel, Sigma) pre-equilibrated with 30 mM Tris-HCl, pH 7.9. The recombinant protein was purified according to the manufacturer’s instructions. The molecular mass and purity of the protein were analyzed by SDS-PAGE after dialysis against 50 mM HEPES-NaOH buffer, pH 7.2. The isocitrate lyase (ICL) concentration was determined spectrophotometrically using a molar extinction coefficient at 280 nm of 64540 M⁻¹.cm⁻¹. Chloroplastic 2-Cys peroxiredoxin (PRX) from *Chlamydomonas* was produced and purified as described in a previous study (19). Recombinant and native HSP70B from *Chlamydomonas* was produced and purified as previously described (20, 21).

**Glutathionylation Treatments**—In vitro glutathionylation assays were performed by treating 0.7 mg/ml HSP70B purified from *Chlamydomonas* or 1.2 mg/ml recombinant HSP70B or 67 μM 2-Cys PRX or 219 μM ICL with 5 mM GSSG for 16 h or with 0.1 mM H₂O₂ plus 0.5 mM GSH for 2 h. Glutathione and H₂O₂ were removed by dialysis before MALDI-TOF mass spectrometry analysis.

**Enzyme Assays**—ICL activity was assayed according to a previous study (22). The reaction mixture contained 50 mM HEPES-NaOH, pH 7.2, 10 mM MgCl₂, 4 mM *three*-DL-isocitrate, 4 mM phenylhydrazine, and enzyme in a final volume of 1 ml. Isocitrate cleavage was measured at 30 °C by the change in absorbance at 324 nm associated with the formation of glyoxylate-phenylhydrazone.

**RESULTS AND DISCUSSION**

We adapted a method developed on human cells (14, 15) for identifying glutathionylated proteins in the green alga *Chlamydomonas* using radiolabeled cysteine. The first step consists of inhibition of protein synthesis in both cytosol and organelles, followed by the addition of radiolabeled cysteine that can enter...
To characterize the effect of a 1 mM sub-lethal concentration of diamide, a thiol-specific oxidant, known to promote efficiently glutathionylation in non-photosynthetic eukaryotes without reactive oxygen species production, is added to per-}


diamide, we measured the pools of total and oxidized glutathione (GSSG) and the GSH/GSSG ratio was only slightly decreased (Fig. 1) indicating that Chlamydomonas cells undergo a moderate oxidative stress during the treatment, leading to cell adaptation but not to cell death.

**Identification of S-Thiolated Proteins**—Following protein extraction, two-dimensional electrophoresis was performed, and radio-labeled proteins were revealed by fluorography (Fig. 2). Control gels were treated with DTT to remove the radiolabeled S-thiol adducts (Fig. 2, B and D). The two Coomassie-stained gels exhibit very similar patterns (Fig. 2, A and B), whereas fluorography reveals important differences (Fig. 2, C and D). Indeed, several proteins are radiolabeled, and this label disappears or is strongly decreased for most proteins after DTT treatment, consistent with the presence of S-thiol adducts on these proteins. Three proteins are labeled with and without DTT treatment (spots 3, 4, and 10 in Fig. 2, C and D). These proteins could require enzymatic deglutathionylation that is not mimicked by DTT, although it cannot be completely ruled out that this labeling is due to residual protein synthesis. All radiolabeled proteins were identified by peptide mass fingerprinting using protein databases as well as Chlamydomonas expressed sequence tag and genome sequences for one of them. This allowed the identification of 25 different targets involved in various cellular processes (Table 1). Among these proteins, 18 are predicted to be localized in chloroplasts, 2 in the cytosol, 2 in flagella, 1 in mitochondria, and 1 in the endoplasmic reticulum. The identification of mainly chloroplastic proteins among S-thiolation targets is consistent with the idea that the chloroplast is the main site of reactive oxygen species production in the light. Indeed, glutathionylation could play an important role in regulating protein activity in this subcellular compartment in the light and thereby allow adaptation of the cell metabolism to different light intensities and qualities (23). Because glutathione represents >95% of total non-protein thiols, glutathionylation is considered to be the prevalent S-thiolation reaction in cells. Moreover, because an increase in glutathione synthesis is observed during the stress treatment, the glutathione pool will be significantly radiolabeled due to the incorporation of [35S]cysteine (Fig. 1). However, the method we have used to detect S-thiolated proteins did not allow discriminating between proteins modified by S-cysteinylated or by S-glutathionylated. Consequently, we will refer to the modified proteins as S-thiolated proteins, although it is likely that most of them have been glutathionylated. Consistent with a modification by S-thiolation, all the proteins identified possess at least one cysteine. Moreover, in most cases, some of the cysteines present in Chlamydomonas proteins are either conserved in other photosynthetic organisms or present in all organisms from bacteria to eukaryotes (supplemental Fig. S1). This could reflect a similar behavior of these proteins, suggesting that glutathionylation could play an important role in the regulation of their activity and/or in their protection.
**TABLE 1**

S-Thiolation targets identified in *C. reinhardtii*

| Accession numbers correspond to NCBI database numbers. Scores correspond to the values given by the Mascot software with the number of matched peptides in parentheses. The sequence coverage values were calculated by the same software as the ratio of the sequence covered by the matched peptides to the sequence of the unprocessed protein. For chloroplastic proteins, these values correspond to the mature form of the protein as predicted by using ChloroP software. Loc, putative subcellular localization; P, plastidial; M, mitochondrial; C, cytosolic; ER, endoplasmic reticulum; F, flagella. Cys, total number of cysteines in the *Chlamydomonas*sequence/number of conserved cysteines in plants/conserved cysteines in animals and/or bacteria when applicable (when not applicable, na is written). Spot, number of spots on Fig. 2 (A and C) corresponding to the proteins identified from the gels shown. Other spots were identified from basic pH range two-dimensional gels. Redox target indicates whether a homologue of the protein has been previously reported to be a (putative) target of TRX (T), GRX (G), and/or S-thiolation (S) (all organisms considered were reviewed in Michelet et al. (6)).

### Proteins

| Gene name | Accession number | Score | Sequence coverage | MM/pl | Loc | Cys | Spot | Redox target |
|-----------|-----------------|-------|-------------------|-------|-----|-----|------|-------------|
| **Photosynthesis** | | | | | | | | |
| Phosphoglycerate kinase | PGK1 | XP_001699523 | 206 (16) | 39 | 43/6.5 | P | 3/2/2 | T, G, S |
| Ribose-5-phosphate isomerase | RIPI | XP_001692016 | 176 (14) | 60 | 25/5.7 | P | 4/2/0 | T |
| OEF1 | PSBO | XP_001694699 | 194 (14) | 41 | 26/5.0 | P | 3/2/na | 8 T |
| Magnesium chelatase Chil | CHL11 | XP_001691232 | 292 (23) | 48 | 40/5.2 | P | 3/3/na | 5 T |
| **Chaperones** | | | | | | | | |
| HSP70B | | XP_001696432 | 269 (26) | 37 | 68/5.0 | P | 3/na | 1 T, G, S |
| Cpn60 o su | CPN60A | XP_001703692 | 221 (19) | 36 | 58/5.1 | P | 3/0/- | 2 T, G |
| Cpn20 | CPN20 | XP_001703710 | 161 (14) | 56 | 21/6.2 | P | 2/0/- | 14 T |
| FKBP 16-3 | FKB16-3 | XP_001692929 | 139 (13) | 48 | 15/5.4 | P | 4/2/- | 13 T |
| FKBP 20-2 | FKB20-2 | XP_001493547 | 118 (8) | 30 | 19/7.9 | P | 6/2/- | T |
| Calreticulin | CRT2 | XP_001898961 | 205 (16) | 43 | 46/4.5 | P | 2/2/2 | 10 |
| **Oxidative stress** | | | | | | | | |
| 2-Cys PRX | PRX1 | XP_001696611 | 152 (10) | 67 | 22/5.2 | P | 2/2/2 | 15 T, G, S |
| 2-Cys PRX | PRX2 | XP_001699660 | 171 (10) | 64 | 22/5.5 | C | 3/2/2 | 11 T, G, S |
| **Amino acid metabolism** | | | | | | | | |
| Aspartate amino transferase | AST1 | XP_001695040 | 263 (21) | 47 | 44/8.9 | P | 7/2/1 | T |
| **Energy/ATP metabolism** | | | | | | | | |
| ATP synthase β chain | ATPB | NP_958414 | 237 (24) | 48 | 52/5.2 | P | 2/1/0 | 3 |
| Inorganic pyrophosphatase | IPY1 | XP_001702577 | 193 (15) | 37 | 27/5.2 | P | 4/0/0 | 7 T, S |
| **Acetate assimilation** | | | | | | | | |
| ICLb | ICL1 | XP_001695331 | 130 (14) | 29 | 46/5.9 | M | 4/1/2 | T |
| **Pentose phosphate pathway** | | | | | | | | |
| Transaldolase | TAL1 | XP_001690070 | 170 (19) | 42 | 42/8.8 | C | 6/0/3 | T |
| **Translation** | | | | | | | | |
| RNA-binding protein RB38 | RB38 | XP_001703164 | 185 (16) | 46 | 42/5.9 | P | 1/-1/- | 6 |
| RNA-binding protein RB60 | RB60 | XP_001701755 | 258 (21) | 41 | 58/4.8 | P | 4/4/4 | 16 T, G |
| Elongation factor Tu | tafP | NP_953862 | 143 (14) | 39 | 46/4.5 | P | 1/1/1 | 4 T, G |
| **Flagella-associated proteins** | | | | | | | | |
| FAP103 (nucleoside diphosphate kinase) | FAP103 | XP_0011698246 | 178 (13) | 64 | 17/6.2 | F | 1/0/0 | 9 T, G, S |
| FAP162 | FAP162 | XP_001702217 | 232 (16) | 55 | 19/4.5 | F | 1/-1/- | 12 |
| **Lipid metabolism** | | | | | | | | |
| Acetyl-CoA: dmitrogen carrier | BCC1 | XP_001700442 | 140 (10) | 23 | 18/5.5 | P | 1/1/- | 18 |
| **Unknown function** | | | | | | | | |
| Algae-specific protein | | | | | | | | |
| Plastid lipid-associated protein 10 | PLAP10 | 28:696041-697542 | ND (8) | 41 | 25/9.2 | P | 31/1/7 | 17 |

* The corresponding spots are radiolabeled with or without DTT treatment.
* Identified in a mixture with elongation factor Tu.
* ND, not determined.

Photosynthesis—Phosphoglycerate kinase and ribose-5-phosphate isomerase, two Calvin cycle enzymes, were identified among S-thiolated proteins. They have already been identified as putative TRX targets in photosynthetic organisms (18, 24, 25), suggesting they were redox-regulated. Phosphoglycerate kinase has also been identified as a putative glutaredoxin (GRX) target in plants (26) and as a glutathionylated protein in non-photosynthetic organisms (14). Moreover, the sequence of phosphoglycerate kinase is highly conserved among organisms, including two partially conserved cysteine residues, and its activity has been reported to be affected by thiol derivatization in several organisms, probably because the modification affects the catalytic cysteine (27–29). This suggests that glutathionylation could affect phosphoglycerate kinase activity under oxidative stress conditions. Two other Calvin cycle enzymes have been previously identified as glutathionylation targets: fructose-1,6-bisphosphate aldolase (13) and A4-GAPDH (11). Loss of activity upon glutathionylation has been reported for the latter. Interestingly, glutathionylation of t-type TRX has also been reported (9). This chloroplastic TRX is involved in light regulation of carbon metabolism enzymes, in particular in the activation of Calvin cycle enzymes in the light. Glutathionylation of this protein affects its reduction, and it can consequently affect the regulation of all its targets. All these data suggest that glutathionylation could allow a regulation of the Calvin cycle under oxidative stress conditions (6, 23). TRX f and A4-GAPDH were not identified here among glutathionylated proteins. The two-dimensional gels we have employed, although being adapted to most proteins, do not allow recovery of proteins with a low molecular weight and high pl such as TRX f. The absence of A4-GAPDH may be linked to the use of diamide for the oxidative stress treatment. Indeed, in mammalian cells, GAPDH is the major target for modification following treatment with hydroperoxides, whereas this enzyme is unaffected.
following cellular exposure to diamide (30). We also identified two other proteins involved in photosynthesis. OEE1 has been already identified as a putative TRX target by proteomics in photosynthetic organisms (18, 25). Magnesium chelatase ChlII is a subunit of magnesium protoporphyrin IX chelatase, which catalyzes the insertion of Mg$^{2+}$ into tetrapyrroles. It exhibits an ATPase activity that is activated by TRX f through the reduction of an intramolecular disulfide between two cysteines conserved among photosynthetic organisms (31). The protein contains three strictly conserved cysteines (supplemental Fig. S1).

Chaperones—Several chaperones have been previously shown to be redox-regulated (32–35). For example, the Arabidopsis TRX-like protein TDX can form a complex with an HSP70 chaperone in yeast, and HSP70 is released under oxidative stress (35). Five of the targets identified here are chaperones: two immunophilins of the FKBP-type, Cpn60 α-subunit, Cpn20, and HSP70B. These five chaperones are all located in the chloroplast. Most of them have been previously identified as putative TRX targets in Chlamydomonas (25). FKBP-type immunophilins are chloroplast thylakoid chaperones, which have been shown to be redox-regulated (36, 37). Interestingly, a redox-chaperone network recently identified in E. coli contains homologs of HSP70, Cpn20, and Cpn60 (33). We investigated further HSP70B glutathionylation. This protein is involved in the folding of chloroplast proteins, the assembly/disassembly of VPP1 oligomers, and the protection of photosystem II from damage inflicted by high light (38, 39). HSP70B, purified from Chlamydomonas or recombinant, was subjected to a classic glutathionylation treatment, i.e. GSSG or GSH plus an oxidant (H$_2$O$_2$). The samples corresponding to native algal or recombinant HSP70B were not distinguishable by MALDI-TOF mass spectrometry. Compared with untreated samples, a mass increase was observed for glutathione-treated samples, consistent with the presence of one glutathione adduct per protein. It was completely reversed by DTT treatment (data not shown). Tryptic digestion of glutathionylated HSP70B led to the identification of Cys-349 as the only residue undergoing the modification (Fig. 3). This cysteine residue is shared by different HSP70 proteins in photosynthetic organisms, mainly predicted to be cytosolic (supplemental Fig. S1). Moreover, most HSP70 proteins possess at least one cysteine residue in this part of the primary sequence. According to three-dimensional modeling, Cys-349 is located in domain IIB of the ATPase domain of HSP70, in the middle of an α-helix following the exposed loop essential for the interaction with GrpE-type chaperones (Fig. 4). Consequently, glutathionylation of this cysteine is likely to affect the ATPase activity of HSP70. Recently, glutathionylation of rat HSC70 was found to lead to a 5-fold increase of efficiency in preventing heat-induced protein aggregation, but the residues involved were not identified (40). We also identified calreticulin, a Ca$^{2+}$-binding chaperone involved in Ca$^{2+}$ homeostasis in mammals and in different other cellular processes (reviewed in Ref. 41). Interestingly, this protein has been found to function with the protein disulfide isomerase Erp57, a member of the TRX superfamily, in mammalian cells (42), suggesting again a close link between glutathionylation and the members of the TRX superfamily.

Translation—Three proteins involved in translation have been identified: RB60, RB38, and elongation factor Tu. RB60 is a protein disulfide isomerase targeted to the chloroplast (43) and is a TRX-dependent translational regulator (25, 44). RB60 and RB38 are part, with RB47 and RB55, of the RB complex, which binds to the 5′-untranslated region of the psbA mRNA and thereby regulates the translation of the D1 protein of photosystem II in response to light (45). It has been shown that the activation of psbA translation in the light by the RB complex requires a reduction step, presumably involving TRX but also an oxidation step mediated by an unknown mechanism (46, 47). The identification of RB60 and RB38 in this study indicates that the oxidation step might involve glutathionylation. Another translation factor, chloroplastic elongation factor Tu, identified in many studies as a TRX target (25, 48–50), and more recently as a GRX target (26), has also been identified in this study. This
protein contains only one cysteine residue that is strictly conserved in all organisms, from bacteria to human. The modification of this cysteine by glutathionylation may therefore represent a general mechanism for protection and/or regulation of this translation factor.

Oxidative Stress—PRXs are thiol-dependent peroxidases that can be reduced by different electron donors, including TRX and GRX (51). Both cytosolic and chloroplastic 2-Cys PRX have been found to be S-thiolation targets in this work. In vitro studies previously suggested that Arabidopsis 2-Cys PRX could undergo glutathionylation (12). In addition, many PRXs have been identified among glutathionylated proteins in mammals (14, 15, 52–55). We investigated chloroplastic 2-Cys PRX glutathionylation by mass spectrometry and found that the classic glutathionylation treatments led to a dimer/monomer switch, as well as an increase of mass of the monomers consistent with the presence of two glutathione adducts (Fig. 5). Because only two cysteines, essential for PRX activity, are present in this protein (supplemental Fig. S1), glutathionylation is likely to inhibit protein activity. Because chloroplastic and cytosolic 2-Cys PRX share a very close sequence similarity, cytosolic PRX is probably affected in the same way. Poplar PRXIIIB, a type II PRX, was reported to undergo dimer dissociation upon glutathionylation (56). Glutathionylation could thus constitute a mechanism of regulation of the oligomerization/activity of PRX. Moreover, the similarity of the results obtained for poplar PRXIIIB and Chlamydomonas 2-Cys PRX suggests the existence of a redox dependent dimer/monomer switch in the PRX family.

Acetate Assimilation—ICL, a key enzyme of acetate assimilation in Chlamydomonas and prokaryotes and involved in germination of oil rich seeds in plants, has been identified as an S-thiolation target. Chlamydomonas ICL possesses four cysteines. Cysteine 178 is the strictly conserved catalytic cysteine while a second cysteine, C301, is conserved among acetate assimilating organisms (supplemental Fig. S1). Mass spectrometry analysis following glutathionylation treatments revealed the presence of two glutathione adducts (Fig. 6B). Tryptic digests showed that the targeted residues were the two non conserved cysteines, C165 and C247 (data not shown). One can thus wonder if the glutathionylation of ICL affects the activity of the protein. Activity measurements revealed that glutathionylated ICL exhibits almost no activity compared with the unmodified ICL (Fig. 6C). DTT treatment, which allows ICL deglutathionylation (Fig. 6D), led to ~80% recovery of activity (Fig. 6C). Moreover, we have previously shown that ICL activity was activated by TRX-dependent reduction in Chlamydomonas total extracts (25). This suggests that the assimilation of acetate is tightly redox-regulated. Further studies will be required to understand the precise molecular mechanisms underlying the complex regulation of ICL by TRX and glutathionylation.

Energy/ATP Metabolism—Chloroplastic inorganic pyrophosphatase, which we previously identified as a putative target of TRX in Chlamydomonas (25), appears to undergo S-thiolation in vivo. Several subunits of chloroplastic, mitochondrial, and vacuolar ATP synthases have also been identified among putative TRX targets in photosynthetic and non-photosynthetic organisms (24, 25, 49, 57–60). Activation of chloroplastic ATP synthase in the light by TRX reduction of a disulfide on the γ-subunit has been well studied (57). However, the β-subunit of chloroplastic ATP synthase has been identified as an S-thiolated protein in this study suggesting an additional redox regulation of this complex.

Lipid Metabolism—The first committed step of fatty-acid bio-

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**FIGURE 5.** Glutathionylation of chloroplastic 2-Cys peroxiredoxins. MALDI-TOF spectra of chloroplastic 2-Cys peroxiredoxin before (A) or after (B) glutathionylation treatments (5 mM GSSG or 0.5 mM GSH plus 0.1 mM H₂O₂).

**FIGURE 6.** Glutathionylation of isocitrate lyase and effect on protein activity. MALDI-TOF spectra of whole isocitrate lyase before (A) or after (B) glutathionylation treatments (5 mM GSSG for 16 h or 0.1 mM H₂O₂ plus 0.5 mM GSH for 2 h). Activity of ICL, glutathionylated (white bars) or not (black bars) has been assayed before or after treatment with 10 mM DTT for 30 min (C). The data are presented as percent maximal activity ± S.D. corresponding to five replicates.
synthesis is catalyzed by acetyl-CoA carboxylase (ACCase), a heterotetramer composed of biotin carboxylase, biotin carboxyl carrier protein, and carboxyltransferase α and β subunits (61, 62). ACCase is a well established target of TRX regulated by redox regulation of a key disulfide bridge linking the α and β subunits of carboxyltransferase (63–65). Here we identified a distinct subunit of ACCase, acetyl-CoA biotin carboxyl carrier, as an S-thiolated protein in Chlamydomonas. Moreover, it was also shown that the biotin carboxylase subunit of ACCase is glutathionylated in vivo in Arabidopsis cells (12). These results suggest that all subunits of ACCase are redox-regulated but through distinct molecular mechanisms.

**Flagellar Proteins and Algae-specific Proteins**—Several S-thiolated proteins identified here are specific to algae including two flagellar proteins, FAP103 and FAP162, identified in the flagella proteome (66). FAP103 is related to nucleoside diphosphate kinases, proteins reported to be part of different signaling pathways in photosynthetic and non-photosynthetic organisms. However, the only cysteine present in this protein is not conserved, even if nucleoside diphosphate kinases present in other organisms possess a cysteine residue in the same part of the primary sequence (supplemental Fig. S1). Nevertheless, nucleoside diphosphate kinases have been identified as TRX (49, 58, 67), GRX (26), or glutathionylation targets (53, 68) in photosynthetic organisms and/or non-photosynthetic organisms suggesting a redox regulation of these proteins.

**Other Pathways**—We also identified S-thiolated proteins participating in amino acid metabolism and the pentose phosphate pathway, two processes previously suggested to be regulated by TRX (reviewed in Refs. 6 and 23). Once again, this result underscores the interplay between redox regulations mediated by TRX and glutathionylation.

**CONCLUSION**

The results presented here constitute the first large scale in vivo proteomic analysis of S-thiolated proteins in a photosynthesizing organism. This indicates that glutathionylation could constitute, as in mammals, a mechanism of redox regulation under oxidative stress conditions in photosynthetic organisms. We identified 25 S-thiolated proteins, involved in 10 different processes. The glutathionylation of three of these targets was confirmed in vitro. Glutathionylation of these proteins could constitute a mechanism of protection against irreversible oxidation but is also likely to affect the activity of several proteins, as demonstrated here for ICL or suggested for HSP70B and chloroplastic 2-Cys PRX. This study constitutes a first step toward the completion of the proteome of glutathionylation targets in photosynthetic cells. Additional studies either in vivo or in vitro, using different types of oxidative stress and detection techniques, will allow increasing the number of known targets in the oncoming years. In mammals, diamide and H2O2 led to the identification of different glutathionylated proteins (69). Thus, it will also probably be necessary to use different oxidative stress conditions, whether physiological or not, to get a more complete picture of the diversity of glutathionylated proteins in photosynthetic organisms. To determine the role of glutathionylation in cells, the exact effect of this post-translational modification on the targets identified will need to be investigated. Because some of these proteins have already been identified as putative targets of TRX and/or GRX (Table 1), it will be of particular interest to determine if these proteins can indeed be redox regulated by the three different systems and the precise underlying molecular mechanisms.

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