ORIGINAL RESEARCH COMMUNICATION

Insight into Protein S-nitrosylation in *Chlamydomonas reinhardtii*

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Abstract

Aims: Protein S-nitrosylation, a post-translational modification (PTM) consisting of the covalent binding of nitric oxide (NO) to a cysteine thiol moiety, plays a major role in cell signaling and is recognized to be involved in numerous physiological processes and diseases in mammals. The importance of nitrosylation in photosynthetic eukaryotes has been less studied. The aim of this study was to expand our knowledge on protein nitrosylation by performing a large-scale proteomic analysis of proteins undergoing nitrosylation *in vivo* in *Chlamydomonas reinhardtii* cells under nitrosative stress. **Results:** Using two complementary proteomic approaches, 492 nitrosylated proteins were identified. They participate in a wide range of biological processes and pathways, including photosynthesis, carbohydrate metabolism, amino acid metabolism, translation, protein folding or degradation, cell motility, and stress. Several proteins were confirmed *in vitro* by western blot, site-directed mutagenesis and activity measurements. Moreover, 392 sites of nitrosylation were also identified. These results strongly suggest that S-nitrosylation could constitute a major mechanism of regulation in *C. reinhardtii* under nitrosative stress conditions. **Innovation:** This study constitutes the largest proteomic analysis of protein nitrosylation reported to date. **Conclusion:** The identification of 381 previously unrecognized targets of nitrosylation further extends our knowledge on the importance of this PTM in photosynthetic eukaryotes. The data have been deposited to the ProteomeXchange repository with identifier PXD000569. *Antioxid. Redox Signal.* 21, 1271–1284.

Introduction

BY GOVERNING PROTEIN properties and functions, post-translational modifications (PTMs) are central mechanisms in cell signaling and adaptation processes (10). Among them, S-nitrosylation, consisting of the covalent binding of the membrane-diffusible free radical nitric oxide (NO) to a protein cysteine thiol moiety, is now recognized to be involved in numerous physiological processes and diseases in mammals (15).

NO production has also been reported in other organisms such as plants where it has been detected in different tissues or subcellular compartments, including plastids, peroxisomes, or mitochondria (5, 16). Whereas NO is mainly produced by NO synthases (NOS) in animals, the presence of such arginine-dependent NO-producing systems in plant

Innovation

Protein nitrosylation is a post-translational modification (PTM) that plays a major role in cell signaling but whose importance in photosynthetic eukaryotes has been less studied than in animals. This study reports the largest proteomic analysis of protein nitrosylation reported to date. Using two complementary proteomic approaches, 492 nitrosylated proteins and 392 sites of nitrosylation (cysteines) were identified. Overall, 381 proteins correspond to previously unrecognized targets of nitrosylation. These results considerably extend our knowledge on the importance of this PTM in photosynthetic eukaryotes.

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cells is still a matter of debate (16). To date, NOS homologue could only be characterized in the alga *Ostreococcus tauri* (13), whereas in other algae and land plants, nitrite and nitrate reductase (NR) seem to be the most potent NO-producing system (5, 16).

In plants, NO has been associated with different cellular processes, including cell death, stomatal closure, floral transition, or root development, and increased levels of S-nitrosylation were observed in response to a huge variety of stresses, such as low or high temperature, high salinity, pathogen attack, or drought (4, 5, 56). Besides the possibility to directly nitrosylate protein thiols, NO can also react with the most abundant intracellular cysteine-containing tripeptide glutathione (GSH; γ -L-glutamyl-L-cysteinylglycine) to form S-nitrosogluthathione (GSNO). GSNO is actually considered the main mobile NO reservoir of the cell and a major trans-nitrosylating agent. GSNO concentration is controlled by GSNO reductase (GSNOR), a widely conserved enzyme catalyzing the reduction of GSNO to oxidized glutathione (GSSG) and ammonia and modulating indirectly the level of nitrosylated proteins (34).

Compared to mammals, only a limited number of studies aiming to analyze the diversity of S-nitrosylated proteins in photosynthetic organisms have been reported. They were performed after biotic or abiotic stresses in *Arabidopsis thaliana*, *Brassica juncea*, citrus and maize; after NO donor treatment in *A. thaliana*, *Kalanchoe pinnata*, *Antiaris toxicaria*, and *Symphytum tuberosum*; in organelles such as *Arabidopsis* mitochondria and pea peroxisomes; or by using a NO accumulation mutant in rice [reviewed in Astier and Lindermayr (3)]. Altogether, these studies lead to a repertoire of ~200 S-nitrosylated proteins and putative sites of modifications for 25%–30% of them.

Mounting evidence suggests the existence of an intricate cross talk between GSH and NO. Besides nitrosylation, GSNO can also promote protein glutathionylation (17, 58). Conversely, GSH plays a crucial role in the control of nitrosylation and its reaction with protein nitrosothiols can lead either to glutathionylation or to denitrosylation (9, 60). Recently, we

have identified 225 glutathionylated proteins and their putative sites of modifications (57) in *Chlamydomonas reinhardtii*, a unicellular eukaryotic green alga that constitutes a photosynthetic model organism well suited for redox biology studies (26). Even if *Chlamydomonas* has been reported to produce NO (44) and its genome encodes a GSNOR, the occurrence of S-nitrosylation in *Chlamydomonas* was not reported.

In the present study, we report a very large-scale proteomic analysis of proteins undergoing nitrosylation *in vivo* in *Chlamydomonas* cells under nitrosative stress. A total of 492 nitrosylated proteins and 392 nitrosylation sites were identified. Several targets were confirmed *in vitro* by western blot and activity assays. In the case of isocitrate lyase, site-directed mutagenesis demonstrated that S-nitrosylation of cysteine 178 reversibly inhibits protein activity. These results further extend our knowledge on the importance of nitrosylation in photosynthetic eukaryotes.

Results

Development of an optimized method for the detection of S-nitrosylation in *Chlamydomonas* cells

The ability of GSNO to trigger S-nitrosylation was investigated for different GSNO concentrations or incubation times (Fig. 1) using the biotin switch technique (BST). This method consists of a three-step reaction for (i) blocking of all free cysteine residues with alkylating agents, (ii) reduction of S-nitrosylated residues with ascorbate, and (iii) biotin-labeling of previously S-nitrosylated cysteine residues with HPDP-biotin, a thiol-specific dithiothreitol (DTT)-reducible biotinylating agent. After nonreducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and blotting, the presence of S-nitrosylated proteins can be followed by immunodetection with anti-biotin antibodies. The signal followed a dose-dependent profile and was abolished by the addition of DTT (Fig. 1A). The 20 kDa band detected in all samples likely corresponds to an endogenously biotinylated protein previously described in *Chlamydomonas* (57). At 2 mM GSNO, the signal increased in a time-dependent manner

FIG. 1. GSNO treatment induces S-nitrosylation in *Chlamydomonas* cells. (A) Nitrosylation is dependent on GSNO concentration. *Chlamydomonas* cells were incubated in the presence of increasing concentrations of GSNO (0, 0.1, 1, 2, and 5 mM) for 15 min. Protein extracts were then subjected to the BST allowing the detection of S-nitrosylated proteins by immunoblotting with anti-biotin antibodies. The reversibility of the biotin signal was assessed by incubating biotinylated proteins with 1 mM DTT for 30 min before gel loading (right lane). (B) Time-dependent nitrosylation after GSNO treatment. *Chlamydomonas* cells were treated with buffer ("Ctrl" lane) or with 2 mM GSNO for 5, 10, 15, or 30 min. Protein extracts were analyzed by BST and immunoblotting as in (A). CBB, Coomassie Brilliant Blue loading control; BST, biotin switch technique; DTT, dithiothreitol; GSNO, S-nitrosogluthathione.

and was maximal and stable after 10 min suggesting that S-nitrosylation occurred rapidly (Fig. 1B). Only cells under nitrosative stress (2 mM GSNO, 15 min) exhibited a strong signal, whereas no signal was detected on untreated cells, cells treated with H₂O₂ or glutathione (GSH or GSSG), DTT-treated controls, or when ascorbate reduction was omitted (Fig. 2A and Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/ars). These results indicate that the BST allows specific detection of numerous proteins undergoing nitrosylation in *Chlamydomonas*.

Identification of nitrosylated proteins and modification sites by complementary mass spectrometry approaches

Chlamydomonas cultures were treated with 2 mM GSNO for 15 min, protein extracts were subjected to the BST, and biotinylated proteins were purified by streptavidin–agarose affinity chromatography. Retained proteins were selectively eluted using DTT and separated by SDS-PAGE (Fig. 2B). Control untreated cells were subjected to the same workflow. Numerous proteins could be detected in the eluate derived from GSNO-treated cells, whereas none was detected in the control underscoring the high specificity of the enrichment step. Moreover, the affinity chromatography step was highly efficient as the flow-through was nearly fully depleted of biotinylated proteins (Supplementary Fig. S2). Whole lanes of GSNO and control samples from two biological replicates were cut into 7 to 10 bands before in-gel digestion with trypsin and analyzed by nLC-MS/MS. Using the MASCOT software with stringent validation criteria (two different peptides having a peptide false discovery rate [FDR] below 1%), 318 proteins were identified in GSNO-treated samples against only 2 proteins in control samples (Supplementary Table S1). In the latter, one protein was only identified in control samples while ATP synthase CF1 beta subunit was present in both conditions but with much more peptides and a higher sequence coverage in GSNO-treated samples and was therefore maintained in the list. The presence of these pro-

teins as traces in control samples likely reflects their abundance. Among the 318 proteins identified, only 24 lacked cysteines and were omitted from the final list. They may correspond to copurified proteins or to wrongly predicted proteins due to erroneous or incomplete gene models.

To get insight into putative S-nitrosylated sites and also cover more deeply the proteins regulated by S-nitrosylation *in vivo*, we used the SNO-site identification (SNOSID) method that is based on the same principle as BST but includes a tryptic digestion before the affinity chromatography step (19). Therefore, only S-nitrosylated peptides are eluted and can be identified by tandem mass spectrometry. GSNO or control samples were prepared under the conditions described above for BST (2 mM GSNO, 15 min) and subjected to the SNOSID approach. A total of 502 peptide sequences were matched by the search engine (Supplementary Table S1). After manual curation, 79 ambiguous peptide sequences were excluded. The remaining 423 peptide sequences correspond to 392 putative nitrosylation sites on 304 proteins (Supplementary Table S2). Seven distinct nitrosylated sites (β -tubulin 2 C354, RubisCO LSU C427, cysteine endopeptidase C311, elongation factor 2 C372, ribosomal protein S5 C161, triose phosphate isomerase C245, and vacuolar ATP synthase subunit E C200) were previously found in *Arabidopsis* (12). In the latter study, much less nitrosylation sites were identified (56 vs. 392 in the present study). This difference is most probably linked to the use of distinct biotin labeling and elution strategies. Indeed, Fares and colleagues employed ICAT labeling and acid elution, a strategy that may have decreased the elution efficiency compared to the HPDP-biotin labeling and the DTT elution used in *Chlamydomonas*.

To date, this study constitutes the most in-depth analysis of S-nitrosylation in photosynthetic eukaryotes as the two strategies allowed *in fine* identification of 492 putative nitrosylated proteins with a global overlap of 21.5% demonstrating the complementarity of the two methods (Supplementary Fig. S3). This low overlap is likely linked to the fact that SNOSID allows to recover nitrosylated peptides that

FIG. 2. S-nitrosylation profile after GSNO treatment of *Chlamydomonas* cells. (A) S-nitrosylation profile of *Chlamydomonas* proteins. *Chlamydomonas* cells were grown in TAP medium and then treated for 15 min with 2 mM GSNO or with an equal volume of H₂O (Ctrl). After incubation, proteins were extracted and subjected to the BST. Control experiments were conducted by replacing ascorbate with NaCl. Proteins were then separated by nonreducing SDS-PAGE and analyzed by anti-biotin western blot. (B) Elution profile of streptavidin affinity purified proteins. *Chlamydomonas* cells were treated as in (A), and biotinylated proteins were loaded on a streptavidin column and eluted with 5 mM DTT. Eluted proteins were separated by SDS-PAGE and silver stained. SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TAP, Tris acetate phosphate.

FIG. 3. Functional annotation of proteins undergoing S-nitrosylation in *Chlamydomonas* cells exposed to GSNO-mediated nitrosative stress. Proteins were classified according to the Kyoto Encyclopedia of Genes and Genomes for *Chlamydomonas* and using a limited number of Gene Ontology annotation levels. They were represented as nodes in a NO-centered network using Cytoscape software (47). Nodes are labeled either with the Gene name or with the UniprotKB identifier. Blue links indicate proteins identified by the BST, orange links by SNOSID, and red links by both methods. Proteins are grouped according to the most important metabolic pathways or cellular processes. A more precise description of protein functions is provided by additional color codes. NO, nitric oxide; SNOSID, SNO-site identification.

are not detected with whole proteins and that BST allows protein identification even if the cysteine-containing peptide is not detected by the mass spectrometer. A similarly low overlap between different methods aimed at identifying redox targets of thioredoxin (TRX) by mass spectrometry has been previously reported (35). The identified proteins are

involved in a wide variety of cellular processes and metabolic pathways, including photosynthesis, carbohydrate metabolism, amino acid metabolism, translation, protein folding or degradation, cell motility, and stress responses, and 17% have unknown functions (Fig. 3). Some pathways appear to be specifically targeted. For example, all 11 enzymes of the

FIG. 4. Kinetics of the *in vivo* denitrosylation after GSNO treatment. (A) BST assay. *Chlamydomonas* cells were treated with 2 mM GSNO for 15 min, washed, and then resuspended in TAP medium and allowed to grow for additional 30, 45, 165, or 240 min. Thereafter, S-nitrosylation levels were analyzed by the BST and visualized by anti-biotin western blots. Untreated cells were used as control (Ctrl). (B) Saville–Griess assay. *Chlamydomonas* cells were treated for 15 min with buffer or 2 mM GSNO, pelleted, resuspended in TAP medium, and allowed to grow for additional 15, 30, or 75 min. Thereafter, cell cultures were centrifuged, washed twice with TAP medium, and total extracts were prepared by three freeze/thaw cycles in liquid nitrogen. S-nitrosothiol contents in protein extracts were determined by the Saville–Griess method. Data are represented as mean percentage $\pm$ SD ($n=3$). **Differences were significant at $p<0.01$ according to the Student's *t*-test.

Calvin–Benson cycle and almost all enzymes of the iron assimilation pathway were identified. The list includes $\sim 54\%$ (114 proteins) of the proteins previously identified as nitrosylated in plants (Supplementary Table S3), a high coverage considering the variety of stresses and species previously analyzed. Some of these proteins are established targets of nitrosylation, such as cytosolic aldolase (50), mitochondrial glycine decarboxylase (42), or cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (22, 60). Overall, 381 identified proteins are previously unrecognized targets of nitrosylation in photosynthetic eukaryotes. This further extends our knowledge on the importance of this PTM in these organisms.

GSNO induces a transient and reversible nitrosative stress

To ensure that the high number of proteins identified reflects the sensitivity of our proteomic approaches rather than the effect of an excessive and irreversible nitrosative stress, we monitored the transitory nature of GSNO-triggered S-nitrosylation and its effect on cell growth and viability. After GSNO treatment (2 mM, 15 min), we visualized the kinetic of the *in vivo* denitrosylation either by western blot using the BST (Fig. 4A) or by the Saville–Griess assay that allows the detection of low- and high-molecular-weight nitrosothiols (Fig. 4B). Protein S-nitrosylation and the nitrosothiol content were

FIG. 5. Effect of GSNO treatment on the growth and viability of *Chlamydomonas* cells. (A) Cell growth. *Chlamydomonas* cells were grown in TAP medium, and two sets of cultures (treated with GSNO or with TAP) were analyzed simultaneously. When cell density reached 5×10^6 cells.mL⁻¹, cells were harvested by centrifugation and resuspended in TAP medium in absence (Control) or in the presence of 2 mM GSNO. After 15 min incubation, cells were pelleted and resuspended in TAP medium at the same cell density (5×10^6 cells.mL⁻¹) and allowed to grow for additional hours (10, 30, and 50 h). Cell density was determined using the Malassez hemacytometer. Cell densities are represented as mean $\pm$ SD ($n=3$). (B) Spot assay after GSNO treatment. *Chlamydomonas* cells were grown in the TAP medium as described above. When cell density reached 2×10^6 cells.mL⁻¹, cells were harvested by centrifugation and treated for 15 min with increasing concentrations of GSNO (2, 20, 50, 50, and 100 mM) or H₂O₂ (2 and 10 mM). After pelleting, cells were resuspended in TAP medium at the same cell density (2×10^6 cell.mL⁻¹). Five microliters of serial dilutions (from 1 to 1/1000) in TAP medium was spotted on TAP agar plates and grown for 10 days at 25°C under continuous light ($80 \mu\text{E.m}^{-2}.\text{s}^{-1}$).

maximal just after the GSNO treatment but decreased rapidly within 1 h indicating that the nitrosative stress is transient. In addition, no significant growth delay was observed after GSNO treatment (Fig. 5A). Moreover, treatments with 2 mM GSNO did not induce cell death, whereas cell viability was compromised above 20 mM (Fig. 5B). The intracellular concentration of GSNO remains unknown but is likely significantly lower than the external concentration. Consistently, as determined by the Saville–Griess assay, only 10% of the GSNO added in cell cultures was consumed during the treatment. Overall, these data indicate that the GSNO treatment induces a mild, transient, and reversible nitrosative stress.

The GSNO treatment induces S-nitrosylation on specific cysteine targets

The high number of proteins identified in *Chlamydomonas* compared to previous BST-based studies raises questions about nitrosylation specificity. In *Chlamydomonas* (genome assembly version 5.3.1), 18,161 of the 19,526 proteins contain at least one cysteine. This suggests that under our conditions, GSNO does not induce indiscriminate nitrosylation of all cysteine residues but seems rather specific of a small subset (2.7%) of cysteine-containing proteins. Nevertheless, one can wonder whether accessible cysteines, which constitute a small subset of total cysteine residues, may all undergo nitrosylation. To further examine this issue experimentally, we used three different purified recombinant TRXs as model proteins. In addition to the active-site disulfide, AtTRXf1, CrTRXm, and CrTRXh1 contain one partially accessible, one fully accessible, or no additional cysteines, respectively (Supplementary Fig. S4). The second extra cysteine of CrTRXm is fully buried. Upon GSNO treatment, only AtTRXf1 was nitrosylated after 30 min while a prolonged incubation was required to detect a likely artifactual low level of nitrosylation for CrTRXm. These results are consistent with the ability of AtTRXf1 to undergo glutathionylation (38) and with the fact that cysteine accessibility is not considered a sufficient criterion for selective S-nitrosylation that rather depends on the cysteine environment (21, 37). These results also reinforce the idea that the mild nitrosative stress we employed is not likely to induce artifactual modifications but rather targets specific cysteines.

Bioinformatic exploitation of MS data

Our large data set of putative S-nitrosylated sites and their flanking sequences were submitted to the Motif-X program (46) to identify linear motifs that may explain the specificity of GSNO-induced nitrosylation. We found five statistically significant motifs ($p \leq 10^{-6}$) representing altogether 42% of the data set (371 sequences). Remarkably, all linear motifs involved a lysine at position +9 ($n=35$), +7 ($n=23$), –11 ($n=24$), –13 ($n=30$), and –14 ($n=42$) relative to the modified cysteine (Supplementary Fig. S5). This suggests that S-nitrosylated cysteines have a weak, positionally non-specific preference for lysines in the surrounding of the modification as previously suggested (32). Such motifs cannot be used for the prediction of nitrosylation sites, but their identification reinforces the importance of concerted acid–base catalysis where lysines could facilitate the deprotonation of thiols, thereby increasing the cysteine reactivity (21). Since lysines are found at different positions in the motifs and

FIG. 6. S-nitrosylation of recombinant NADP-MDH, ICL, and IPMDH in the presence of GSNO. Recombinant NADP-MDH, ICL, and IPMDH from *Chlamydomonas reinhardtii* and ATG8 from *Saccharomyces cerevisiae* were incubated with GSNO (2 mM, 15 min) or buffer (H₂O) with or without prior incubation with 100 mM of IAM. The reversibility of the reaction was assessed by the treatment with 10 mM DTT for 30 min. After treatment, recombinant proteins were subjected to the BST and S-nitrosylation was visualized by anti-biotin western blot. ATG8 has no cysteine and was used as a control. IAM, iodoacetamide; NADP-MDH, NADP-dependent malate dehydrogenase.

are not directly in the vicinity of modified cysteines, the tertiary structure must be important to provide such acid–base catalysis (37). For the 217 sites with no identified motif, a lysine or another residue allowing the acid–base catalysis may be located away in the primary structure but brought in the vicinity of the cysteine through the tertiary structure. Some cysteines may also undergo nitrosylation through other mechanisms, such as metal-catalyzed nitrosylation or protein-driven transnitrosylation.

GSNO transnitrosylates 3 candidate proteins in vitro

The *Chlamydomonas* enzymes isocitrate lyase (CrICL), NADP-dependent malate dehydrogenase (CrNADP-MDH), and isopropylmalate dehydrogenase (CrIPMDH) participate in acetate assimilation, export of reducing power from the chloroplast and leucine biosynthesis, respectively. These three enzymes were previously identified as regulated by other redox PTMs (disulfide bonds under the control of TRX for IPMDH and NADP-MDH and glutathionylation for ICL). Therefore, confirmation of their nitrosylation would validate these proteins as models to study the complex interplay between nitrosylation and other redox PTMs. The corresponding coding sequences were cloned and used to recombinantly express the proteins in *Escherichia coli*. All three proteins harbored an N-terminal His-Tag and were purified by nickel

FIG. 7. Effect of GSNO on *Chlamydomonas* ICL and its cysteine variants. (A) WT ICL (10 μ M) and (C) its variant C165S/C247S/C301S (3M) (10 μ M) were treated with GSNO (1 mM, 15 min), and the residual activity was assayed. The reversibility of the reaction was assessed by 10 min treatment with 10 mM DTT. Data are represented as mean percentage $\pm$ SD ($n=3$) of the control (Ctrl) activity (buffer, 15 min). (B) WT ICL and its variants C178S and 3M were incubated with GSNO (1 mM, 15 min) and analyzed by BST and anti-biotin western blot. **Differences were significant at $p<0.01$ according to the Student's t -test. WT, wild type.

affinity chromatography. Immunoblots after BST revealed that all 3 purified proteins were transnitrosylated by GSNO (2 mM, 15 min) *in vitro* (Fig. 6). The signal was abolished by DTT or pretreatment with the cysteine-specific alkylating agent iodoacetamide (IAM) indicating that the absence of free cysteine prevents S-nitrosylation. These results confirm the nitrosylation of three identified proteins involved in distinct pathways. Moreover, CrICL was found to be inhibited after GSNO treatment and fully reactivated by DTT (Fig. 7A). The inhibition was dependent on incubation time and GSNO concentration, and no significant protection was

FIG. 8. Kinetics of WT CrICL inactivation by various concentrations of GSNO. Prerduced WT CrICL (10 μ M) was incubated with buffer alone (open circle) or with increasing concentrations of GSNO (0.1 mM, black triangle; 0.2 mM, black circle; 0.5 mM, black diamond). The possible substrate protection was tested by incubating the protein with 0.5 mM GSNO in the presence of 4 mM threo-DL-isocitrate (black square). Aliquots of the incubation mixtures were withdrawn at the indicated times, and the remaining activity was determined. Activities are represented as mean percentage $\pm$ SD ($n=3$) of the initial ICL activity measured before inactivation treatments. CrICL, *Chlamydomonas* isocitrate lyase.

provided by the substrate isocitrate (Fig. 8). Since CrICL can be inhibited by glutathionylation (7) and GSNO is known to induce both nitrosylation and glutathionylation (17, 58), the inhibition was confirmed with [diethylamine NONOate (diazoniumdiolate) diethylammonium salt] (DEA-NONOate) as an alternative and specific NO-donor. The extent of inhibition and the DTT reversibility were comparable to those obtained with GSNO (Fig. 9A), thereby confirming that CrICL inhibition was due to the NO moiety. As MALDI-TOF mass spectrometry failed to detect all cysteine-containing peptides, the site of nitrosylation was investigated by site-directed mutagenesis. CrICL contains four cysteines, and the nitrosylation was dependent on the presence of the strictly conserved catalytic Cys178. Indeed, the nitrosylation signal was abolished by the C178S mutation, whereas the Cys165S/Cys247S/Cys301S triple mutant (3M) behaved as the wild-type (WT) enzyme (Fig. 7B). Moreover, the activity of the triple mutant was comparable to the WT enzyme in terms of inhibition by GSNO or DEA-NONOate and subsequent DTT reactivation (Figs. 7 and 9B). All together, these data strongly suggest that the CrICL activity is reversibly inhibited by nitrosylation of catalytic Cys178.

Discussion

The comprehensive list of 492 nitrosylated proteins identified in *Chlamydomonas* will be extremely useful for understanding the molecular mechanisms underlying the regulation of numerous pathways and processes in photosynthetic eukaryotes and other organisms. This list is of course not exhaustive as numerous proteins are excluded from our analyses, such as proteins not expressed under basal conditions or proteins undergoing nitrosylation through a mechanism independent of GSNO. Proteomic studies are also intrinsically biased toward the identification of abundant proteins, a bias partly limited with the BST approach due to the affinity purification step. Nevertheless, many of the identified proteins are abundant, but this is not true for all protein classes. For example, *Chlamydomonas* contains 10 TRX isoforms, some of which (TRXf1, TRXm, and TRXh1) are highly expressed (26), but the two TRXs found are TRXo and TRXh2, which likely represent the less abundant isoforms. Noteworthy, TRXf1 that undergoes nitrosylation *in vitro* was not detected as nitrosylated *in vivo*. The bias toward abundant proteins may have limited the identification of

FIG. 9. Reversibility of WT CrICL and triple mutant C165S/C247S/C301S treatment in the presence of DEA-NONOate. (A) WT CrICL (10 μ M) and (B) triple mutant C165S/C247S/C301S (3 M, 10 μ M) were treated with 1 mM DEA-NONOate, and after 15 min incubation, the residual activity was assayed. The reversibility of the reaction was assessed by 10 min treatment with 10 mM DTT. Data are represented as mean percentage $\pm$ SD ($n=3$) of WT and mutant activity measured after 15 min incubation with buffer alone (Ctrl). **Differences were significant at $p<0.01$ according to the Student's t -test. DEA-NONOate, [diethylamine NONOate (diazoniumdiolate) diethylammonium salt].

nitrosylated transcription factors that may play an important role in responses to nitrosative stress. Several transcriptional regulators have been reported to be nitrosylated, including the plant NPR1 and TGA1 transcription factors (33), which are involved in plant defense against pathogens but have no ortholog in *Chlamydomonas*. Nevertheless, NO-regulated transcription factors may be present among the 85 proteins of unknown function. Interestingly, numerous targets are involved in protein translation/folding/degradation, suggesting that under nitrosative stress, protein expression might be mainly regulated at the translational and post-translational levels in *Chlamydomonas*.

The list of 392 nitrosylation sites will also be very useful for future studies. As for proteins, the list is not exhaustive as many peptides are not detected by mass spectrometry due to their relative abundance and their physicochemical properties. For example, CrICL Cys178-containing peptide is usually not detected (7) and was therefore not identified by SNOSID, although this residue is the unique site of nitrosylation *in vitro*. However, the three other cysteines were identified by the SNOSID method suggesting that these sites may undergo *in vivo* nitrosylation through other mechanisms mediated by other forms of NO or by transnitrosylating proteins. Similar results were obtained with GAPDH whose catalytic cysteine is known to be nitrosylated but for which 4 sites were detected by the SNO resin-assisted capture (SNORAC) method (14).

The extensive list of nitrosylation sites obtained in *Chlamydomonas* will also be useful to improve prediction programs. GPS-SNO (54) and iSNO-PseAAC (53) are recent web-based interface programs that aim at predicting S-nitrosylation sites. To compare their prediction efficiency, we built a data set containing the *Chlamydomonas* proteins for which the nitrosylation sites were experimentally determined by the SNOSID approach. This data set was submitted to both prediction programs, which failed to identify correctly the experimental nitrosylated sites in *Chlamydomonas* proteins (Supplementary Table S4). In fact, the stringency of the prediction appeared either too high (GPS-SNO) or too low (iSNO-PseAAC). These results indicate that current prediction programs fail to accurately predict S-nitrosylation sites, at least for *Chlamydomonas* proteins. The availability of large experimental data sets, such as the one reported here,

will certainly be helpful for future improvement and training of such prediction programs.

It should be kept in mind that the nitrosylation of an enzyme involved in a specific pathway does not necessarily imply that this pathway is regulated by nitrosylation. First, it is possible that nitrosylation does not affect the activity of the protein. However, even if it does, as shown here for ICL, the extent of the modification *in vivo* being undetermined, it is possible that nitrosylation only affects a minor pool of the total protein and nitrosylation of this pool may not be limiting for the pathway. Finally, numerous identified proteins may represent moonlighting proteins that, upon nitrosylation, are diverted to new functions unrelated to their established function as shown for GAPDH in mammals (20). Indeed, upon apoptotic stimulation, nitrosylation of this glycolytic enzyme triggers its translocation to the nucleus where it regulates gene expression through several mechanisms, including transnitrosylation of nuclear proteins (25). Plant GAPDH was also shown to undergo nitrosylation and to re-localize to the nucleus under stress condition, but the exact physiological function of the modification remains to be established (22, 51, 60).

Among the 492 nitrosylated proteins identified, numerous were previously identified as glutathionylated (39, 57) or as putative TRX targets (27) (Supplementary Fig. S6). The Calvin-Benson cycle constitutes a typical example of a metabolic pathway regulated by multiple redox PTMs. Indeed, all 11 enzymes of this pathway were found to be modified by glutathionylation (57, 59), nitrosylation (this study) and were also all identified as putative TRX targets (28, 31). These results suggest that enzymes of the Calvin-Benson cycle are regulated by multiple redox PTMs. These modifications may allow a fine-tuning of the Calvin-Benson cycle that could allow a redistribution of the energy (in the form of ATP) and reducing power (in the form of NADPH) within chloroplasts under stress conditions (28, 38). This redistribution may be required transiently to cope with stress conditions. Redox PTMs may also divert these abundant enzymes to new functions unrelated to their metabolic role in carbon metabolism. The only Calvin-Benson cycle enzyme for which a moonlighting function has been reported is ribulose-1,5-bisphosphate carboxylase (Rubisco). Indeed, under oxidative stress, the Rubisco

enzyme comprising eight small subunits (SSU) and eight large subunits (LSU) disassembles into its constituents, and LSU subsequently bind chloroplast mRNAs nonspecifically and form large particles (11, 24, 55). Recently, *Chlamydomonas* LSU but not SSU were shown to accumulate in chloroplast stress granules (cpSGs) under oxidative stress conditions (49). cpSGs are RNA granules related to mammalian stress granules that form during oxidative stress and disassemble during recovery from stress. This study therefore suggested a novel function of Rubisco LSU as an mRNA-localizing and assembly factor of cpSGs (49). This moonlighting function being triggered by oxidative stress conditions, it may likely be regulated by redox PTMs. Indeed, several Rubisco cysteines appear to be reactive and have been suggested to play a role in the regulation of the enzyme activity and stability (1, 2, 40, 41, 48). Since cysteine modifications control several moonlighting functions of cytosolic GAPDH, the same may be true for chloroplastic GAPDH isoforms participating in the Calvin–Benson cycle, which also undergo multiple redox PTMs. Among the hundreds of proteins identified as putative TRX-targets, some may be devoid of TRX-reduced intra- or interprotein disulfides. Indeed, TRX may also participate in the catalysis of denitrosylation (8) and deglutathionylation (6, 18). Therefore, an overlap between TRX-targets and nitrosylated/glutathionylated proteins may reflect the multiple functions of TRXs rather than the regulation of the same proteins by multiple redox PTMs. However, among the 239 putative glutathionylated proteins identified in *Chlamydomonas* (39, 57), 125 were found as nitrosylated. This double identification is consistent with the existence of a strong interplay between nitrosylation and glutathionylation as previously described (59). Unraveling the importance of this cross talk under diverse physiological and growth conditions and characterizing the underlying molecular and structural determinants will certainly constitute a major challenge for future studies.

Materials and Methods

Materials and chemicals

NAP-5 columns were obtained from GE Healthcare. Proteomics grade endoproteinases (Lys-C and Trypsin Gold) were purchased from Promega. HPDP-biotin (N-[6-(Biotinamido)hexyl]-3'-(2'-pyridylthio)propionamide) was purchased from Pierce. All other chemicals or antibodies (monoclonal anti-biotin antibody, B7653; rabbit anti-mouse antibody, A9044) were purchased from Sigma-Aldrich. GSNO and DEA-NONOate concentration in the solution was determined spectrophotometrically using molar extinction coefficients of $920\text{ M}^{-1}\text{ cm}^{-1}$ at 335 nm (GSNO) and $6700\text{ M}^{-1}\text{ cm}^{-1}$ at 250 nm (DEA-NONOate).

Chlamydomonas cultures and protein extraction

Conditions for *Chlamydomonas* cultures and protein extraction were adapted from Zaffagnini *et al.* (57). Briefly, the *Chlamydomonas* D66 cell-wall-less strain (CC-4425 cw nit2-203 mt⁺ strain) was grown in Tris acetate phosphate (TAP) medium under continuous light ($80\text{ }\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at 25°C up to $3\text{--}5\times 10^6$ cells·ml⁻¹. Cultures were then pelleted (4000 g, 10 min), resuspended in 3–5 ml of TAP medium, and subjected

to specific treatments using freshly prepared compounds (GSNO, GSH, GSSG, or H₂O₂). After 15 min incubation, cells were harvested by centrifugation, washed once with TAP medium, centrifuged again, and resuspended in 2 ml of blocking buffer containing 50 mM Tris-HCl, pH 7.9, 1 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaCl, 1% SDS, protease inhibitors (SigmaFast, EDTA free), and cysteine alkylating reagents (20 mM IAM, 20 mM N-ethylmaleimide [NEM]). Total soluble proteins were extracted by three freeze/thaw cycles in liquid nitrogen, and protein extracts were left for an additional 30 min at room temperature under agitation to allow complete free thiols alkylation. After incubation, protein samples were centrifuged (15,000 g, 15 min, 4°C) to remove any insoluble material, and protein concentration was determined by the BCA assay using bovine serum albumin as standard. All treatments were performed away from direct light to prevent artifactual denitrosylation.

Determination of nitrosothiol content

The S-nitrosothiol content in protein extracts was determined by the Saville–Griess method (45). Briefly, samples containing 250 μg of proteins were incubated with 1.08% sulfanilamide (stock solution prepared at 2.7% in 0.4 M HCl) and 0.03% N-(1-naphthyl)-ethylenediamine (stock solution prepared at 1.0% in water) in the presence of 0.1% HgCl₂. Control experiments were performed by omitting HgCl₂ from the samples. After 20 min incubation in the dark, color formation of the azo dye was complete, and the absorbance at 540 nm was recorded spectrophotometrically. The absorbance at 540 nm of control samples (absence of HgCl₂) is linked to the presence of free nitrite in the sample and was subtracted from the absorbance measured in the presence of HgCl₂ to estimate the nitrosothiol content using a GSNO standard curve obtained with the same assay.

Ascorbate reduction and biotin labeling of in vivo S-nitrosylated proteins

Detection of S-nitrosylated proteins from protein extracts was performed using the BST (23) following the procedure described in (60) with minor modifications. Briefly, cells treatment and protein extraction were performed as described above. After alkylation step, samples were precipitated with three volumes of cold acetone at -20°C for 30 min to remove excess of alkylating reagents, centrifuged (15,000 g, 10 min, 4°C), and resuspended at the concentration of 1 mg/ml in TEN buffer (30 mM Tris-HCl pH 7.9, 1 mM EDTA, 100 mM NaCl) supplemented with 1% SDS. Biotinylation of S-nitrosylated proteins was achieved by adding 40 mM ascorbate and 0.25 mM HPDP-Biotin. After 30 min incubation at room temperature in the dark, the excess of ascorbate and HPDP-biotin was removed by acetone precipitation, and precipitated proteins were then resuspended at 1 mg/ml in TEN buffer.

Gel electrophoresis and Western blot analysis

Proteins were separated by nonreducing SDS-PAGE (12%) and electrotransferred onto nitrocellulose membranes using a semidry transblot apparatus (Bio-Rad Laboratories). Membranes were blocked with 5% (w/v) nonfat dry milk in Tris buffer saline solution (TBS) containing 0.1% Tween 20 for 1 h at room temperature and incubated overnight at 4°C with the monoclonal anti-biotin antibody

(dilution 1:5000). Membranes were extensively washed with TBS containing 0.1% Tween 20 before the addition of the secondary peroxidase-conjugated rabbit anti-mouse antibody (dilution 1:10,000) for 1 h at room temperature. Signals corresponding to S-nitrosylated proteins were then visualized by chemiluminescence.

Purification of S-nitrosylated proteins by affinity chromatography (BST method)

Biotinylated proteins were resuspended in TEN buffer, and 200 μ g was loaded onto a 1 ml streptavidin-agarose column preequilibrated with TEN buffer and incubated for 30 min at room temperature. The column was extensively washed with 10 volumes of TEN buffer supplemented with NaCl up to 600 mM and then with 10 volumes of TEN buffer. Finally, the proteins retained on the column were eluted with 2 ml of 10 mM DTT dissolved in TEN buffer. The eluted proteins were concentrated by evaporation using a vacuum concentrator, separated by a short migration (2–3 cm) on a reducing 12% SDS-PAGE, and silver stained. Whole lanes corresponding to GSNO-treated and control (without treatment) samples were excised manually in several bands, destained, and subjected to a manual in-gel digestion with modified porcine trypsin as described in (36).

Purification of S-nitrosylated peptides by affinity chromatography (SNOSID method)

Biotinylated proteins, instead of being resuspended in TEN buffer, were resuspended in 25 μ l of 30 mM Tris-HCl (pH 7.9) containing 8 M urea and digested at 30°C for 18 h with Lys-C endoproteinase in a 1:75 (w/w) enzyme:substrate ratio. Then, 210 μ l of 30 mM Tris-HCl (pH 7.9) were added and the sample were further incubated for 4 h at 30°C in the presence of modified porcine trypsin in a 1:50 (w/w) enzyme:substrate ratio. Before streptavidin-based affinity chromatography, samples were diluted twice with 30 mM Tris-HCl, 1 mM EDTA, and 200 mM NaCl. After dilution, samples were loaded onto a 1-ml streptavidin-agarose column preequilibrated with TEN buffer. The column was extensively washed as described above. The final washing step was performed with 10 volumes of 25 mM ammonium bicarbonate. Elution was achieved by incubation of the streptavidin resin for 5 min with 25 mM ammonium bicarbonate supplemented with 5 mM DTT. The SNOSID method allows precise mapping of the nitrosylation sites even for multiple cysteine-containing peptides since non-nitrosylated cysteines are blocked by alkylating agents inducing a mass shift (+57 Da for IAM or +125 Da for NEM) and are thus distinguishable from S-nitrosylated cysteines that are recovered as free thiols after DTT elution.

Tandem mass spectrometry

Tryptic digests were prepared in 18 μ l of 3% acetonitrile (CH_3CN) containing 0.1% v/v formic acid and analyzed on an LTQ Velos Orbitrap (Thermo Fisher Scientific) coupled to a Proxeon Easy nano-LC reversed phase chromatography system (Thermo Fisher Scientific) using a binary solvent system consisting of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Digests obtained from the BST method were loaded on a Proxeon Easy column C18

(10 cm, 75 μ m i.d., 120 Å), and peptides were separated with a flow rate of 300 nl/min by three successive linear gradients of solvent B from 5% to 25% in 20 min, from 25% to 45% in 40 min, and finally up to 80% in 10 min. Digests obtained from the SNOSID method were loaded on an Acclaim Pepmap C18 (25 cm, 75 μ m i.d., 100 Å), and peptides were separated with a flow rate of 300 nl/min by two successive linear gradients of solvent B from 2% to 35% in 100 min and then up to 80% in 6 min followed by an isocratic step at 80% for 4 min.

Peptides were analyzed on the LTQ Velos Orbitrap (Thermo Fisher Scientific) operating in the data-dependent acquisition mode with survey scans acquired at a resolution of 30,000 (at m/z 400 Da) with a mass range of m/z 400–1800. Fragments were obtained by collision-induced dissociation with a collisional energy of 40%, an isolation width of 2 Da, and a Q activation of 0.250 for 10 ms. MS/MS data were acquired in the linear ion trap in a data-dependent mode in which the 20 most intense precursor ions were fragmented, with a dynamic exclusion of 15 s, an exclusion list size of 500, and a repeat duration of 30 s. The maximum ion accumulation times were set to 100 ms for MS acquisition and 50 ms for MS/MS acquisition.

Data analysis and database searches

Raw Orbitrap data were processed with Proteome Discoverer 1.3 software (Thermo Fisher Scientific) and searched against the NCBI nr database limited to the *C. reinhardtii* taxonomy (15,667 entries) using an in-house Mascot search server (version 2.3.2; Matrix Science). Mass tolerance was set to 10 ppm for the parent ion mass and 0.6 Da for fragments. Up to two missed cleavages per peptide were allowed, and methionine oxidation and cysteine carboxyamidomethylation were taken into account as variable modifications. FDRs were determined by searching against a reversed decoy database, and peptide identifications were filtered at 1% FDR. Nitrosylated proteins from the BST method were validated if they were identified with at least two different peptides passing the peptide FDR filter, whereas nitrosylated cysteine-containing peptides from the SNOSID method passing the peptide FDR filter were only considered for identification after manual inspection of the corresponding MS/MS spectra. For the samples prepared according to the BST method, all peak lists from the same experimental condition (GSNO or Control) were concatenated for database searches with MASCOT. Final protein lists were generated using the multireporting capacity of ProteomeDiscoverer and by concatenating data from GSNO or Control experiments from the biological replicates. For the SNOSID approach, each condition (GSNO or Control) was analyzed in one single LC run and one peak list was generated per run for database searches with the MASCOT engine. The lists of identified proteins were generated with ProteomeDiscoverer. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository (52) with the data set identifier PXD000569.

Production and purification of recombinant proteins

Recombinant WT and mutant *Chlamydomonas* ICL were produced and purified as described in (7). WT recombinant cytosolic TRXh1, chloroplastic TRXm, and NADP-MDH

from *C. reinhardtii*, and yeast ATG8 and chloroplastic TRXf1 from *A. thaliana* were produced and purified as previously reported (29, 30, 38, 43). The coding sequence encoding CrIPMDH was obtained by PCR on the EST clone AV629916 obtained from the Kazusa DNA Research Institute (Chiba, Japan), using a forward primer introducing an *NdeI* restriction site (in bold) at the start codon (bolded): 5'-GCGCTCGC **A⁺TA₁TGCTGAACACTGTGTGCAAGGT**-3' and a reverse primer introducing a *BamHI* restriction site (in bold) downstream of the stop codon: 5'-CAGGG⁺**GATC₁CTTACAGGTGCTTCATCAGCT**-3'. CrIPMDH was cloned in a modified pET-3c vector containing additional codons upstream of the *NdeI* site to express a His-tagged protein with 7 N-terminal histidines. The sequence was checked by sequencing. Recombinant CrIPMDH was produced using the pET-3c-HIS/BL21 expression system. Bacteria were grown in the LB medium at 37°C, and the production was induced with 200 μ M isopropyl- β -D-thiogalactopyranoside overnight at 37°C. Cells were harvested by centrifugation, resuspended in 30 mM Tris-HCl, pH 7.9; 1 mM EDTA; 100 μ g/ml phenylmethanesulphonylfluoride, broken using a French press (6.9×10^7 Pa), and cell debris were removed by centrifugation. The supernatant was applied onto a Ni²⁺ Hitrap chelating resin (HIS-Select[®] Nickel Affinity Gel; Sigma) preequilibrated 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 30 mM Tris-HCl, pH 7.9. The IPMDH concentration was determined spectrophotometrically using a molar extinction coefficient at 280 nm of $27,765 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Inactivation of CrICL by nitrosative treatments

Before each treatment, recombinant WT ICL and its cysteine variants were treated with 20 mM DTT for 1 h at room temperature and then desalted using NAP-5 columns pre-equilibrated with 50 mM HEPES-NaOH buffer, pH 7.6. Inactivation treatments were performed at room temperature by treating 10 μ M of ICL with 1 mM GSNO or DEA-NONOate. At indicated times, aliquots were withdrawn to assay the ICL enzymatic activity monitored as described by Bedhomme *et al.* (7). The reversibility of ICL inactivation (15 min incubation with GSNO or DEA-NONOate) was assessed by measuring the ICL activity after incubation for 10 min in the presence of 10 mM DTT.

Detection of the S-nitrosylation state of recombinant proteins

The S-nitrosylation state of recombinant proteins were analyzed by the BST (see above), after 15 min incubation in the presence of 1 mM GSNO (unless otherwise specified).

Growth and viability assays

Cell density was determined using a Malassez hemacytometer. For spot test analysis, *Chlamydomonas* cultures were prepared as described above. After GSNO or control treatment, cells were pelleted and washed with liquid TAP medium and adjusted to 3×10^6 cells.ml⁻¹. Five-microliter drops of treated cultures were spotted on the solid TAP medium by 10 times successive dilutions. The visual analysis was performed after 10 days of growth under continuous light at 25°C.

Replicates

All experiments were performed at least in triplicate, and the data were represented as mean $\pm$ standard deviation.

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Author Disclosure Statement

No competing financial interests exist

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Abbreviations Used

ATG8	= autophagy-related protein 8
BCA	= bicinechoninic acid
BST	= biotin switch technique
CBB	= Coomassie Brilliant Blue
cpSGs	= chloroplast stress granules
Cr	= <i>Chlamydomonas reinhardtii</i>
CrICL	= <i>Chlamydomonas</i> enzymes isocitrate lyase.
DEA-NONOate	= [diethylamine NONOate (diazoniumdiolate) diethylammonium salt]
DTT	= dithiothreitol
EDTA	= ethylenediaminetetraacetic acid
FDR	= false discovery rate
GAPDH	= glyceraldehyde-3-phosphate dehydrogenase
GSH	= reduced glutathione
GSNO	= S-nitrosoglutathione
GSNOR	= GSNO reductase
GSSG	= oxidized glutathione
HPDP-biotin	= N-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide
IAM	= iodoacetamide
ICL	= isocitrate lyase
IPMDH	= isopropylmalate dehydrogenase
MALDI-TOF	= matrix-assisted laser desorption/ionization–time of flight
NADP-MDH	= NADP-dependent malate dehydrogenase
NEM	= N-ethylmaleimide
NO	= nitric oxide
NOS	= NO synthases

Abbreviations Used (Cont.)

NPR1 = nonexpressor of PR-1 transcription factor

NR = nitrate reductase

PTMs = post-translational modifications

Rubisco LSU = ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit

Rubisco SSU = ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit

SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SNORAC = SNO resin-assisted capture

SNOSID = SNO-site identification

TAP = Tris acetate phosphate

TBS = Tris buffer saline solution

TGA1 = TGA1 transcription factor

TRX = thioredoxin

WT = wild type