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# Mechanisms of Nitrosylation and Denitrosylation of Cytoplasmic Glyceraldehyde-3-phosphate Dehydrogenase from *Arabidopsis thaliana*\*

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**Background:** Plant cytoplasmic GAPDH undergoes nitrosylation and glutathionylation.

**Results:** GSNO triggers mainly nitrosylation of *Arabidopsis* GAPDH, which can be denitrosylated by GSH but not by thioredoxins.

**Conclusion:** The extent of GAPDH nitrosylation is dependent on the [GSH]/[GSNO] ratio but independent of the [GSH]/[GSSG] ratio.

**Significance:** The mechanisms of GAPDH nitrosylation are delineated, and the prominent role of glutathione is established.

Nitrosylation is a reversible post-translational modification of protein cysteines playing a major role in cellular regulation and signaling in many organisms, including plants where it has been implicated in the regulation of immunity and cell death. The extent of nitrosylation of a given cysteine residue is governed by the equilibrium between nitrosylation and denitrosylation reactions. The mechanisms of these reactions remain poorly studied in plants. In this study, we have employed glycolytic GAPDH from *Arabidopsis thaliana* as a tool to investigate the molecular mechanisms of nitrosylation and denitrosylation using a combination of approaches, including activity assays, the biotin switch technique, site-directed mutagenesis, and mass spectrometry. *Arabidopsis* GAPDH activity was reversibly inhibited by nitrosylation of catalytic Cys-149 mediated either chemically with a strong NO donor or by trans-nitrosylation with GSNO. GSNO was found to trigger both GAPDH nitrosylation and glutathionylation, although nitrosylation was widely prominent. *Arabidopsis* GAPDH was found to be denitrosylated by GSH but not by plant cytoplasmic thioredoxins. GSH fully converted nitrosylated GAPDH to the reduced, active enzyme, without forming any glutathionylated GAPDH. Thus, we found that nitrosylation of GAPDH is not a step toward formation of the more stable glutathionylated enzyme. GSH-dependent deni-

troxylation of GAPDH was found to be linked to the [GSH]/[GSNO] ratio and to be independent of the [GSH]/[GSSG] ratio. The possible importance of these biochemical properties for the regulation of *Arabidopsis* GAPDH functions *in vivo* is discussed.

The crucial importance of redox post-translational modifications (RPTMs)<sup>3</sup> in cell signaling and regulation and their association with many diseases is largely recognized (1–7). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) act as signaling molecules to transfer extracellular or intracellular information and elicit specific responses. ROS and RNS can alter the function of proteins by triggering diverse RPTMs on several types of residues (6, 8). Cysteine is the major target of reactive species as its thiol side chain can undergo diverse types of reversible modifications, including oxidation of intra- or interprotein disulfides (SS), nitrosylation (SNO), glutathionylation (SSG), and other types of S-thiolation (e.g. cysteinylolation), sulphydration (SSH), or sulfonylation (SOH). In addition, cysteine residues can be irreversibly oxidized by sulfinylation (SO<sub>2</sub>H) or sulfonylation (SO<sub>3</sub>H). These modifications are mainly controlled by small oxidoreductases of the thioredoxin (TRX) and glutaredoxin (GRX) families.

Nitric oxide (NO) is a highly reactive molecule implicated in many signaling processes. NO can be generated by NO synthases (NOS), which convert L-Arg to NO and L-citrulline (three isoforms of NOS are present in mammals), or by the breakdown of nitrite or other compounds to NO, e.g. through

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<sup>3</sup> The abbreviations used are: RPTM, redox post-translational modification; BST, biotin switch technique; DEA-NO, diethylamine NONOate; GR, glutathione reductase; GSNO, S-nitrosoglutathione; TRX, thioredoxin; ROS, reactive oxygen species; RNS, reactive nitrogen species; BisTris, 2-bis(2-hydroxyethyl)-amino]-2-(hydroxymethyl)propane-1,3-diol; GRX, glutaredoxin; HPDP-biotin, N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide; NTR, NADPH: thioredoxin reductase.

cytochrome *c* oxidase in mitochondria or nitrate reductase in plants (9–11). NO is involved in a post-translational modification named nitrosylation consisting of the formation of nitrosothiols by reaction of NO-related species with protein thiols (12). Several hundred targets of nitrosylation have been identified in bacteria, yeast, plants, and animals, suggesting a role for this modification in many cellular processes (13, 14).

S-Nitrosylation appears to be implicated in a broad spectrum of human diseases, including cancer, diabetes, and several neurodegenerative, cardiovascular, muscular, or respiratory diseases (1, 15–17). Although the enzymes controlling NO production in plants remain to be characterized (18), numerous plant proteins appear to undergo nitrosylation under biotic or abiotic stress conditions (13, 19–22). Nitrosylation appears crucial for plant immune response by controlling the activity and/or subcellular localization of the transcription factors NPR1 (Non-expressor of Pathogenesis-related genes 1) and TGA1 but also the salicylic acid-binding protein 3 (SABP3) (23–25). Nitrosylation also modulates cell death during the hypersensitive response to pathogens through regulation of NADPH oxidase (26) or peroxiredoxin IIE (27).

The extent of nitrosylation of a given cysteine residue is governed by the equilibrium between nitrosylation and denitrosylation reactions. Therefore, protein nitrosylation is dependent on the activity of NO-producing enzymes. Nitrosylation can be triggered chemically by RNS such as the NO radical (NO $\cdot$ ), the nitrosonium cation (NO $^+$ ), the nitroxyl anion (NO $^-$ ), or peroxynitrite (ONOO $^-$ ) or by trans-nitrosylation reactions mediated by small nitrosothiols such as nitrosogluthathione (GSNO) or by other nitrosylated proteins (12, 28, 29). Indeed, several nitrosylated proteins are able to act as cysteine trans-nitrosylases by transferring their NO group to target proteins as shown for hemoglobin (30), thioredoxin (31–33), caspase-3 (34), cyclin-dependent kinase 5 (35), or GAPDH (36). Some proteins also mediate transfer of NO from an intrinsically bound heme iron or other transition metal complexes to cysteine thiols (16). These metal-to-Cys nitrosylases mainly catalyze intramolecular NO group transfer (auto-S-nitrosylation, *e.g.* hemoglobin) or intermolecular reaction with glutathione to form GSNO (*e.g.* cytochrome *c*).

GSNO is the main NO reservoir of the cell and a major trans-nitrosylating molecule. GSNO is formed by reaction of glutathione, the main small soluble thiol of the cell, with RNS. GSNO not only induces protein trans-nitrosylation but also protein glutathionylation (37, 38). GSNO concentration is mainly controlled by GSNO reductase, an enzyme widely expressed in prokaryotes and eukaryotes (19, 39, 40). GSNO reductase catalyzes the NADH-dependent reduction of GSNO and, in the presence of GSH, gives rise to GSSG and ammonia. GSNO reductase controls the cellular levels of GSNO and therefore influences indirectly the level of nitrosylated proteins. Several other enzymes have also been suggested to control GSNO levels, including protein-disulfide reductase (41) and thioredoxin (42, 43). Similarly, flavohemoglobins in bacteria and fungi control NO accumulation and also affect indirectly nitrosylation levels (44–46).

The reactions of denitrosylation are much less documented. Two main mechanisms appear to be involved. The first one

involves reaction with glutathione that leads to GSNO formation that can be subsequently reduced by GSNO reductase. Consistently, the absence of GSNO reductase leads to a strong increase of protein nitrosylation in mice, yeast, and plants (19, 39, 47). Most protein nitrosothiols (75–80%) were shown to be susceptible to reduction by GSH in rat brain extracts or in rat spinal cords (48, 49). The second mechanism involves mammalian cytoplasmic and mitochondrial TRX, which were shown to denitrosylate numerous proteins (43, 50–54). Other enzymes such as protein-disulfide isomerase, xanthine oxidase, carbonyl reductase, glutathione peroxidase, and superoxide dismutase have been suggested to act as denitrosylases but remain to be confirmed (28).

GAPDH, a glycolytic enzyme, is the most widely used model protein to investigate the molecular mechanisms of RPTMs. Indeed, GAPDH contains a highly reactive catalytic cysteine that undergoes multiple types of RPTM, including SNO, SSG, SSH, SOH, SO $_2$ H, or SO $_3$ H. All of these modifications inhibit the glycolytic activity of GAPDH, which can therefore be used to measure precisely the extent and the reversibility of a given RPTM. In animal cells, redox modifications of GAPDH can redirect the protein to new and completely unrelated functions. Indeed, this glycolytic enzyme was demonstrated to play a role in several cellular processes such as receptor-mediated signaling, intracellular membrane trafficking, maintenance of DNA integrity, transcriptional and post-transcriptional regulation, oxidative stress response, and apoptosis (55, 56). Several of these functions involve re-localization of GAPDH to the nucleus and are triggered or regulated by redox modifications of the catalytic cysteine. For instance, upon apoptotic stimulation, nitrosylation of GAPDH triggers its binding to the E3 ubiquitin ligase SIAH1, which possesses a nuclear localization signal and mediates translocation of GAPDH to the nucleus (57). The translocation of the GAPDH-SIAH1 complex to the nucleus ultimately leads to apoptosis through several mechanisms involving not only Siah1-dependent ubiquitination and degradation of nuclear proteins but also GAPDH-mediated regulation of gene expression. Indeed, nuclear GAPDH controls the regulation of nuclear genes such as *p53*, *PUMA*, *BAX*, and *p21* through interaction with the nuclear acetyltransferases p300 and CBP (58). Moreover, nitrosylated GAPDH was also shown to trans-nitrosylate several nuclear proteins (HDAC, SIRT-1, and DNA-PK (36)).

Plants contain several isoforms of GAPDH encoded by *gapA*, *gapB*, and *gapC* genes. Indeed, in addition to glycolytic GAPDH isoforms (cytoplasmic GAPC1, GAPC2, and plastidic GAPCP1, GAPCP2), A $_2$ B $_2$ -GAPDH and A $_4$ -GAPDH are located in chloroplast stroma and participate in the Calvin-Benson cycle. These chloroplastic GAPDH are likely regulated by different types of RPTMs. We have recently employed two different forms of GAPDH from *Arabidopsis thaliana* (hereafter referred to as *Arabidopsis*) as tools to analyze the enzymes and mechanisms controlling glutathionylation/deglutathionylation reactions. Notably, A $_4$ -GAPDH and GAPC1 are very sensitive to oxidation and are rapidly irreversibly inactivated by sulfinylation and/or sulfonylation in the presence of ROS such as H $_2$ O $_2$  (59, 60). However, both types of enzymes can be protected from irreversible oxidative inactivation by glutathiony-



lation of the catalytic cysteine, a modification occurring by reaction of GSH with the sulfenic acid formed on the catalytic cysteine of A<sub>4</sub>-GAPDH and GAPC1 after primary oxidation by H<sub>2</sub>O<sub>2</sub> (59, 60). Because of the stable nature of glutathione-protein-mixed disulfide, glutathionylated forms of A<sub>4</sub>-GAPDH and GAPC1 were used to delineate the mechanisms involved in deglutathionylation reactions catalyzed by oxidoreductases like GRXs and TRXs (60–62).

By contrast, the molecular mechanisms of plant GAPDH nitrosylation remain much less documented. Several proteomic studies have identified cytoplasmic and chloroplastic GAPDH from *Arabidopsis* as potential targets of nitrosylation (27, 63), and the modification was confirmed *in vitro* for the *Arabidopsis* GAPC isoforms (64). More generally, the mechanisms of nitrosylation and denitrosylation remain poorly studied in plants. In addition, it is not known whether plant TRXs can catalyze protein denitrosylation.

In this study, we have employed glycolytic GAPDH isoform 1 from *A. thaliana* (AtGAPC1) as a tool to investigate the molecular mechanisms of nitrosylation and denitrosylation using a combination of approaches, including activity assays, the biotin switch technique, site-directed mutagenesis, and mass spectrometry. Nitrosylation reactions were induced chemically with a strong NO donor or by trans-nitrosylation with GSNO. The ability of GSNO to trigger both nitrosylation and glutathionylation was investigated, and the site of modification was demonstrated experimentally. The ability and the efficiency of glutathione and different types of plant TRXs to catalyze protein denitrosylation were also examined. Nitrosylation and glutathionylation of GAPC1 were found to be alternative and not inter-convertible RPTMs of the same catalytic cysteine. Denitrosylation of GAPC1 was specifically performed by GSH and found to be directly controlled by the [GSH]/[GSNO] ratio but independent from the [GSH]/[GSSG] ratio.

## EXPERIMENTAL PROCEDURES

**Materials and Enzymes**—PD-10 and NAP-5 columns were obtained from GE Healthcare. *N*-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide (HPDP-biotin) was purchased from Pierce. GSNO (nitrosoglutathione) and DEA-NONOate (DEA-NO, diethylamine NONOate (diazoniumdiolate) diethylammonium salt) were purchased from Sigma, and their concentration in solution was determined spectrophotometrically using molar extinction coefficients of 920 M<sup>-1</sup> cm<sup>-1</sup> at 335 nm (GSNO) and 6700 M<sup>-1</sup> cm<sup>-1</sup> at 250 nm (DEA-NO). All other chemicals were obtained from Sigma. Glutathione reductase (GR) and 3-phosphoglycerate kinase from *S. cerevisiae* were purchased from Sigma. Recombinant wild-type TRXh1 from *C. reinhardtii* was prepared as described previously (65). Recombinant NADPH TRX reductase b from *A. thaliana* and poplar recombinant h-type TRXs (isoforms h 1, h 2, h 3, and h 5) were expressed and purified as described previously (66–69).

**Expression and Purification**—Cytoplasmic GAPC1 from *A. thaliana* and the cysteine mutants GAPC1-C149S (C149S) and GAPC1-C153S (C153S) were expressed and purified according to Ref. 60. The subunit molecular mass and purity of the proteins were analyzed by SDS-PAGE (12% gel) and Coomassie Blue staining after desalting on PD-10 columns equilibrated

with 50 mM potassium phosphate buffer (pH 7.5). The protein concentration (on a subunit basis) of recombinant proteins was determined spectrophotometrically using a molar extinction coefficient at 280 nm of 40,910 M<sup>-1</sup> cm<sup>-1</sup>. The resulting homogeneous proteins were stored at –20 °C.

**Activity Assays**—GAPC1 activity was monitored spectrophotometrically at 340 nm and 25 °C by following the reverse (gluconeogenic) reaction assay. The reaction was measured in an assay mixture containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM MgCl<sub>2</sub>, 3 mM 3-phosphoglycerate, 5 units/ml of *Saccharomyces cerevisiae* 3-phosphoglycerate kinase, 2 mM ATP, and 0.2 mM NADH.

**Inactivation of GAPC1 by GSNO and DEA-NO Treatments**—Before each treatment, wild-type GAPC1 and its cysteine variants were reduced in the presence of 10 mM dithiothreitol (DTT) for 30 min at room temperature (25 °C). DTT was subsequently removed by desalting on NAP-5 columns equilibrated with 50 mM BisTris buffer (pH 7.0). Reduced GAPC1 (2.0 μM or 78 μg/ml final concentration) was incubated in 50 mM BisTris buffer (pH 7.0) with different concentrations of GSNO (0.1, 0.5, and 1 mM) or DEA-NO (0.1, 0.5, and 1 mM) in the presence or absence of 0.14 mM NAD<sup>+</sup>. At the indicated times, an aliquot of the sample (5–10 μl) was withdrawn for the assay of enzyme activity. The reversibility of GAPC1 inactivation (10 min of incubation with 0.5 mM GSNO or 0.5 mM DEA-NO) was assessed by measuring GAPDH activity after incubation for 15 min in the presence of 20 mM DTT.

**MALDI-TOF Mass Spectrometry**—Wild-type GAPC1 and the C149S mutant were treated for 30 min either with 1 mM GSNO or with 50 μM H<sub>2</sub>O<sub>2</sub> and 0.5 mM GSH. The MALDI-TOF mass spectrometry analyses were performed before and after incubation with 20 mM DTT for 30 min. The samples were mixed with a saturated solution of sinapinic acid in 30% acetonitrile containing 0.3% trifluoroacetic acid, deposited onto the sample plate, and allowed to dry. Mass determination of WT and C149S GAPC1 was performed as described previously (70).

**Reactivation of Nitrosylated GAPC1**—Nitrosylated GAPC1 (GAPC1-SNO) was prepared by incubating the enzyme for 30 min in the presence of 1 mM DEA-NO at room temperature and then desalted on a NAP-5 column equilibrated with 50 mM Tris-HCl (pH 7.5) and 1 mM EDTA. Reactivation treatments of GAPC1-SNO were performed in Tris-HCl (pH 7.5) and 1 mM EDTA with 20 mM DTT or in the presence of TRXh isoforms supplemented with 0.22 μM *A. thaliana* NTRb and 0.2 mM NADPH. Control samples were analyzed under similar conditions by omitting TRXs. The reactivation of GAPC1 was also carried out in the presence of 2 mM GSH at different pH values (7.0, 7.5, and 7.9) or in the presence of 2 mM GSH (pH 7.5) alone or supplemented with 0.5 mM GSSG or with the GR system (6 μg/ml yeast GR and 0.2 mM NADPH). At the indicated times, aliquots (5–10 μl) were withdrawn to assay enzyme activity.

**Biotin Switch Technique (BST) to Analyze GAPC1 Nitrosylation/Denitrosylation**—The extent of GAPC1 nitrosylation was assessed by the biotin switch assay adapted from Ref. 71. After nitrosylation or denitrosylation treatments, proteins (1 mg/ml) were precipitated with 4 volumes of cold acetone at –20 °C during 30 min and pelleted by centrifugation at 4 °C for 5 min at

15,000  $\times$  g. The pellet was resuspended in TENS buffer (30 mM Tris-HCl (pH 7.9), 1 mM EDTA, 100 mM NaCl, and 1% SDS) supplemented with a mixture of alkylating reagents (10 mM iodoacetamide, 10 mM *N*-ethylmaleimide), to allow blocking of free thiols. After 30 min of incubation at 25 °C under shaking, the samples were precipitated as described above to remove unreacted alkylating reagents. After resuspension in TENS buffer, proteins were incubated in the presence of 40 mM ascorbate and 1 mM HPDP-biotin for 30 min. This step allows reduction of *S*-nitrosylated cysteines and their derivatization with biotin. Proteins were precipitated to remove unreacted labeling compounds, pelleted by centrifugation as described above, and resuspended in TENS buffer. All steps were performed in darkness until proteins were biotinylated with HPDP-biotin. After final precipitation, proteins were resuspended and quantified using the bicinchoninic acid assay. Proteins were then separated by nonreducing SDS-PAGE, transferred onto nitrocellulose membranes, and analyzed by Western blotting using a primary anti-biotin antibody (1:5,000 dilution; Sigma) and an anti-mouse secondary antibody coupled to peroxidase (1:10,000 dilution; Sigma). Signals were visualized by enhanced chemiluminescence, as described previously (60). A mirror SDS-polyacrylamide gel was prepared from the same samples and stained with Coomassie Brilliant Blue to assess equal loading. All BST assays included a negative control where ascorbate was omitted to prevent reduction of *S*-nitrosothiols and subsequent biotinylation. This control without ascorbate allows us to assess the efficiency of the initial thiol-blocking step.

**Reactivation of GSNO-treated GAPC1 in the Presence of Variable GSH Amounts**—GAPC1 (5  $\mu$ M) was treated with 2 mM GSNO in 50 mM Tris-HCl (pH 7.5) and 1 mM EDTA, and after 30 min of incubation, various amounts of GSH were added to achieve the following [GSH]/[GSNO] ratios: 25, 10, 1, and 0.1. After 10 min of incubation, an aliquot of the samples was withdrawn for the assay of enzyme activity monitored as described above.

**Reactivation of Nitrosylated GAPC1 in the Presence of Various [GSH]/[GSSG] and [GSH]/[GSNO] Ratios**—GAPC1-SNO was prepared as described above and then desalted on a NAP-5 column equilibrated with 50 mM Tris-HCl (pH 7.5) and 1 mM EDTA. Reactivation treatments were carried out by incubating GAPC1-SNO (5  $\mu$ M) in 50 mM Tris-HCl (pH 7.5) and 1 mM EDTA in the presence of 2 mM GSH supplemented with variable amounts of GSSG (0.02, 0.08, 0.2, and 2 mM). After 10 min of incubation, an aliquot of the samples was withdrawn for the assay of enzyme activity monitored as described above.

**Redox Titrations of GAPC1 in the Presence of Different [GSH]/[GSNO] or [GSH]/[GSSG] Ratios**—Reduced GAPC1 (5  $\mu$ M) was incubated in 50 mM Tris-HCl (pH 7.5) and 1 mM EDTA in the presence of different [GSH]/[GSNO] or [GSH]/[GSSG] ratios at a total concentration of 2 mM. After reaching the equilibrium (30 min), aliquots were withdrawn to assay enzyme activity monitored as described above.

**Replicates**—All the results reported are representative of at least three independent experiments and expressed as mean  $\pm$  S.D.

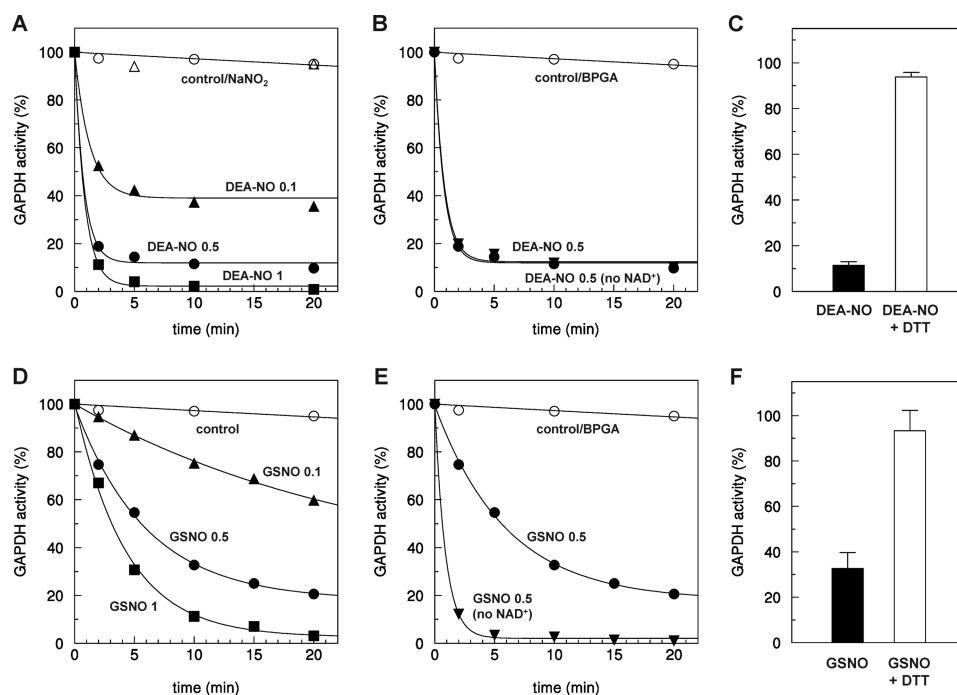
## RESULTS

**GAPC1 Is Reversibly Inhibited by DEA-NO and GSNO**—In *A. thaliana*, cytoplasmic glyceraldehyde-3-phosphate dehydrogenase is represented by two isozymes, GAPC1 (At3g04120) and GapC2 (At1g13440), that are 98% identical and most probably share common biochemical properties. We have recombinantly expressed and purified GAPC1 from *A. thaliana*, an enzyme strictly specific for NADH and containing a strictly conserved catalytic cysteine (60). Although this catalytic cysteine is the 155th residue of *Arabidopsis* GAPC1 sequence, it is named Cys-149 here for congruence with the first solved GAPDH structure (from *Bacillus stearothermophilus*, Protein Data bank code 2dbv (72)) in which catalytic cysteine is numbered Cys-149.

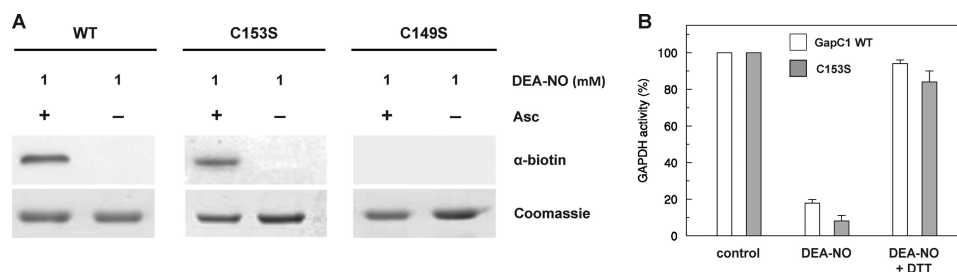
To investigate the regulation of GAPC1 by nitrosylation, the purified enzyme was incubated with different types of NO donors. DEA-NONOate (DEA-NO), dissolved in 10 mM NaOH, behaves as a strong chemical NO donor upon transfer to neutral pH. Typical DEA-NO decomposition rates of  $28.8 \pm 2.3$   $\mu$ M/min were optically determined in 0.2 mM DEA-NO solutions at neutral pH. In the presence of DEA-NO at pH 7.0, GAPC1 activity was strongly inhibited, although the activity remained high in the buffer alone or in the presence of sodium nitrite (NaNO<sub>2</sub>), a product of the spontaneous reaction of NO with O<sub>2</sub>, commonly used for GSNO synthesis (Fig. 1A). The rate and extent of inhibition were dependent on DEA-NO concentration, and GAPC1 was fully inactivated after 5 min of incubation with 1 mM DEA-NO. Lower concentrations of DEA-NO caused rapid but only partial inhibition of GAPC1 (Fig. 1A), probably reflecting the rapid consumption of NO by oxygen. The cofactor NAD, which was present in all incubation media, did not protect GAPC1 from DEA-NO-dependent inhibition (Fig. 1B). By contrast, the substrate 1,3-bisphosphoglycerate, which covalently binds to catalytic Cys-149, allowed full protection of the enzyme suggesting that Cys-149 may be the target of DEA-NO (Fig. 1B). Reaction of DEA-NO with cysteine residues can lead to *S*-nitrosylation, a modification that can be reversed by the strong chemical reductant DTT. Consistently, the inhibition caused by 10 min of incubation with 0.5 mM DEA-NO was nearly completely reversed by DTT (Fig. 1C).

We also analyzed the effect of the trans-nitrosylating agent GSNO on GAPC1 activity. GSNO was found to inhibit GAPC1 activity, and the inactivation was dependent on GSNO concentration (Fig. 1D). The inactivation appeared slower with GSNO compared with DEA-NO. This lower efficiency was most probably due to the presence of the NAD cofactor, bound to the active site, which partially protected the enzyme from GSNO-dependent inhibition. Indeed, in the absence of NAD, 0.5 mM GSNO led to complete inhibition of GAPC1 after 5 min of incubation. This partial protection is consistent with a higher steric hindrance of the NAD cofactor for GSNO compared with NO (Fig. 1E). GAPC1 incubated for 10 min in the presence of 0.5 mM GSNO could be largely reversed by DTT. All these results are consistent with both DEA-NO and GSNO triggering inhibition of GAPC1 through *S*-nitrosylation.

**GAPC1 Is Nitrosylated on Catalytic Cys-149**—The BST allows replacement of labile nitrosothiols by a biotin moiety



**FIGURE 1. Inactivation of AtGAPC1 by DEA-NO and GSNO.** *A*, time-dependent inactivation of GAPC1 in the presence of DEA-NO. Reduced GAPC1 (2  $\mu$ M) was incubated either with buffer (open circles) or NaNO<sub>2</sub> (open triangles) as control or with DEA-NO at different concentrations (0.1 mM, closed up-triangles; 0.5 mM, closed circles; 1 mM, closed squares). *B*, kinetics of inactivation of GAPC1 in the presence of 0.5 mM DEA-NO supplemented or not with 0.14 mM NAD<sup>+</sup>. *C*, reversibility of DEA-NO treatment. Reduced GAPC1 was incubated for 10 min with 0.5 mM DEA-NO (black bar). The reversibility of GAPC1 inactivation was assessed by incubation for 15 min in the presence of 20 mM DTT (white bars). NADH-dependent GAPC1 activity was then determined. *D*, time-dependent inactivation of GAPC1 in the presence of GSNO. Reduced GAPC1 (2  $\mu$ M) was incubated with buffer as control (open circles) or with GSNO at different concentrations (0.1 mM, closed up-triangles; 0.5 mM, closed circles; 1 mM, closed squares). *E*, kinetics of inactivation of GAPC1 in the presence of 0.5 mM GSNO supplemented or not with 0.14 mM NAD<sup>+</sup>. *F*, reversibility of GSNO treatment. Reduced GAPC1 was incubated for 10 min with 0.5 mM GSNO (black bar). The reversibility of GAPC1 inactivation was assessed by incubation for 15 min in the presence of 20 mM DTT (white bars). NADH-dependent GAPC1 activity was then determined. *A*, *B*, *D*, and *E*, aliquots of the incubation mixtures were withdrawn at the indicated time points, and the remaining NADH-dependent activity was determined. Data represent the mean percentage ( $n = 3$ ) of maximal control activity. *C* and *F*, NADH-dependent activities of GAPC1 are expressed as mean percentages ( $n = 3$ ) of the GAPC1 activity measured after 10 min of incubation under control conditions. *BPGA*, 1,3-bisphosphoglycerate. Except for *C* and *F*, S.D. was omitted for clarity.



**FIGURE 2. Nitrosylation of GAPC1 and its cysteine variants in the presence of DEA-NO.** *A*, WT GAPC1, C153S, or C149S mutants (25  $\mu$ M each) were treated for 30 min in the presence of 1 mM DEA-NO, and nitrosylation was visualized using the biotin switch technique followed by anti-biotin Western blots as described under "Experimental Procedures." The Coomassie Brilliant Blue staining of the gel shows equal loading in each lane. *Asc*, ascorbate. *B*, WT GAPC1 or C153S mutants (25  $\mu$ M) were treated with 1 mM DEA-NO, and after 30 min the residual NADH-dependent activity was assayed. The reversibility of the reaction was assessed by 15 min of treatment with 20 mM DTT. Data are represented as mean percentage  $\pm$  S.D. ( $n = 3$ ) of the GapC activity measured after 30 min of incubation under control conditions.

(71). BST consists of three major steps. 1) All free thiols are initially blocked. 2) Nitrosothiols are specifically reduced with ascorbate. 3) Newly exposed thiols are derivatized with HPDP-biotin. Omission of ascorbate in the second step can be used as control. BST allows direct visualization of protein nitrosylation by Western blot using anti-biotin antibodies.

We employed BST to confirm GAPC1 nitrosylation. GAPC1 was incubated for 30 min with 1 mM DEA-NO and analyzed by BST followed by anti-biotin Western blots after separation by nonreducing SDS-PAGE. For wild-type (WT) GAPC1, a strong nitrosylation signal dependent on the presence of ascorbate was

observed (Fig. 2*A*). *Arabidopsis* GAPC1 only contains two cysteines that are both strictly conserved, catalytic Cys-149 and the closely located Cys-153. Cys-149 is absolutely required for GAPDH catalysis, which implies the acylation of the thiolate Cys-149 by the substrate as an essential step of the catalytic cycle (73). However, Cys-153 has apparently no role in catalysis, despite its close proximity with catalytic Cys-149 (60).

BST was used to analyze the nitrosylation of WT GAPC1 compared with the site-specific mutants C149S and C153S. Whereas no signal could be detected with the C149S mutant, strong and comparable signals were detected with the WT



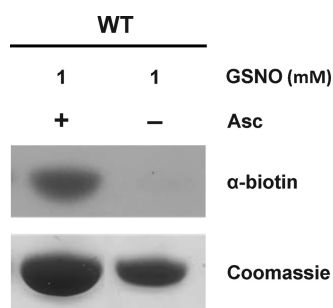


FIGURE 3. **Nitrosylation of GAPC1 in the presence of GSNO.** GAPC1 (25  $\mu$ M) was treated (30 min) with 1 mM GSNO, and nitrosylation was visualized using the biotin switch technique followed by anti-biotin Western blots as described under "Experimental Procedures." The Coomassie Brilliant Blue staining of the gel shows almost equal loading in each lane. Asc, ascorbate.

enzyme and the C153S mutant (Fig. 2A). The C149S variant is "virtually" inactive (60) and could not be used for activity assays. By contrast, activity assays were performed on the WT enzyme and the C153S mutant. As shown in Fig. 2B, both WT GAPC1 and the C153S mutant could be inhibited by DEA-NO and reactivated by DTT. Because the C149S mutant is not nitrosylated and the C153S GAPC1 cannot be distinguished from the WT enzyme in BST and activity assays, we conclude that catalytic Cys-149 is the only target of nitrosylation in WT GAPC1.

**GSNO Inhibition of GAPC1 Activity Is Essentially Due to Trans-nitrosylation**—GSNO can react with cysteine residues to induce not only nitrosylation but also glutathionylation. GAPC1 activity is known to be reversibly inhibited by glutathionylation of catalytic Cys-149 through a mechanism involving reaction of GSH with Cys-149 sulfenate generated by reaction of Cys-149 thiolate with  $\text{H}_2\text{O}_2$  (60). As shown in this study for nitrosylated GAPC1, the inhibition of GAPC1 activity by glutathionylation can be reversed by DTT (60). Therefore, it is not possible to distinguish nitrosylation from glutathionylation based on activity measurements because both modifications inhibit GAPC1 activity and are reversed by DTT.

The ability of GSNO to induce GAPC1 nitrosylation was analyzed using BST and anti-biotin Western blots (Fig. 3). After incubation of GAPC1 for 30 min in the presence of 1 mM GSNO, a strong signal, which is absent without ascorbate, was observed. This suggests that GAPC1 undergoes nitrosylation in the presence of GSNO. However, this does not exclude the possibility that GAPC1 also undergoes glutathionylation concomitantly with nitrosylation. Indeed, with the BST assay being qualitative but not quantitative, it is not possible to determine whether the nitrosylation signal detected corresponds to the total pool of GAPC1 molecules present in the samples or only to a fraction of this pool. It is possible that GSNO induces a partial nitrosylation of GAPC1 molecules (even a minor fraction) and leads to glutathionylation of the remaining GAPC1 enzymes. This situation would be consistent with the BST and the activity assays reported above because GAPC1 would be fully and reversibly inhibited by GSNO due to both nitrosylation and glutathionylation.

The ability of GSNO to trigger GAPC1 glutathionylation was investigated by MALDI-TOF mass spectrometry. Glutathionylation typically results in a 305-Da shift of the mass of the protein that can be usually observed by MALDI-TOF mass spec-

trometry. By contrast, the labile SNO bond is lost during the laser-induced ionization process (74), and therefore the nitrosylated protein is not distinguishable from its unmodified form. MALDI-TOF analysis was performed on WT GAPC1 and the C149S mutant after incubation with 1 mM GSNO for 30 min (Fig. 4). The same proteins were also treated with 50  $\mu$ M  $\text{H}_2\text{O}_2$  and 0.5 mM GSH, conditions known to trigger GAPC1 glutathionylation. After GSNO treatment of WT GAPC1, a minor peak (39,268 Da), shifted by  $\sim 305$  Da compared with the unmodified/nitrosylated protein, was observed (Fig. 4A, upper and middle panels). The control glutathionylation treatment induced a shift of the main peak of WT GAPC1 to the same size of 39,268 Da corresponding to the glutathionylated form (Fig. 4A, lower panel). In both cases, the shift could be reversed by DTT treatment. This reversible mass increase is consistent with the formation of one glutathione adduct per GAPC1 monomer. This indicates that GSNO may induce glutathionylation of GAPC1, although much less efficiently than treating with  $\text{H}_2\text{O}_2$  and GSH (glutathionylation treatment). Integration of the area of the minor peak detected after GSNO treatment indicated that glutathionylated GAPC1 represents less than 10% of the total GAPC1 pool. Therefore, the strong inhibition exerted by GSNO on GAPC1 activity (Fig. 1) must depend on effects other than glutathionylation, i.e. nitrosylation (Fig. 2). By contrast with WT GAPC1, the spectra recorded for unmodified or GSNO-treated C149S mutants were comparable. A similar spectrum was also measured after the glutathionylation treatment of C149S (Fig. 4B), confirming that this mutant could not be glutathionylated.

Altogether, the results reported above indicate the following: (i) GAPC1 activity is fully inhibited by GSNO; (ii) the inhibition is fully reversed by DTT; (iii) GAPC1 is nitrosylated on Cys-149 based on the BST assay, and (iv) GSNO triggers glutathionylation of the same cysteine in a small fraction of GAPC1, as revealed by mass spectrometry. Therefore, it can be concluded that GSNO triggers both trans-nitrosylation and glutathionylation of GAPC1 catalytic Cys-149, with nitrosylated GAPC1 representing by far the major final product of the reaction.

**GAPC1 Can Be Denitrosylated by GSH but Not by Thioredoxin**—Two main mechanisms, involving either GSH or TRX, have been proposed to control protein denitrosylation reactions (28). We therefore investigated the contribution of these two mechanisms in the control of GAPC1 denitrosylation. GAPC1 was nitrosylated with DEA-NO and desalted to remove unreacted NO donors. In these conditions, the activity of GAPC1-SNO was inhibited by 80% and fully recovered in the presence of 20 mM DTT (Fig. 5A). The partial residual activity in these samples compared with previous assays (Fig. 1) was reminiscent of the desalting step and was most probably linked to changes of protein/NO donor ratio and to the instability of the nitrosothiol bond (e.g. photolysis, trace metals, etc.) during desalting. We first investigated the ability of different cytoplasmic h-type TRXs from both poplar and *Chlamydomonas reinhardtii* to catalyze GAPC1 denitrosylation. DTT, even at low concentrations, could not be used as a reductant for TRXs due to its fast reaction with GAPC1-SNO. Therefore, we rather used a physiological reducing system for TRXs composed of NADPH and *A. thaliana* NADPH-thioredoxin reductase b

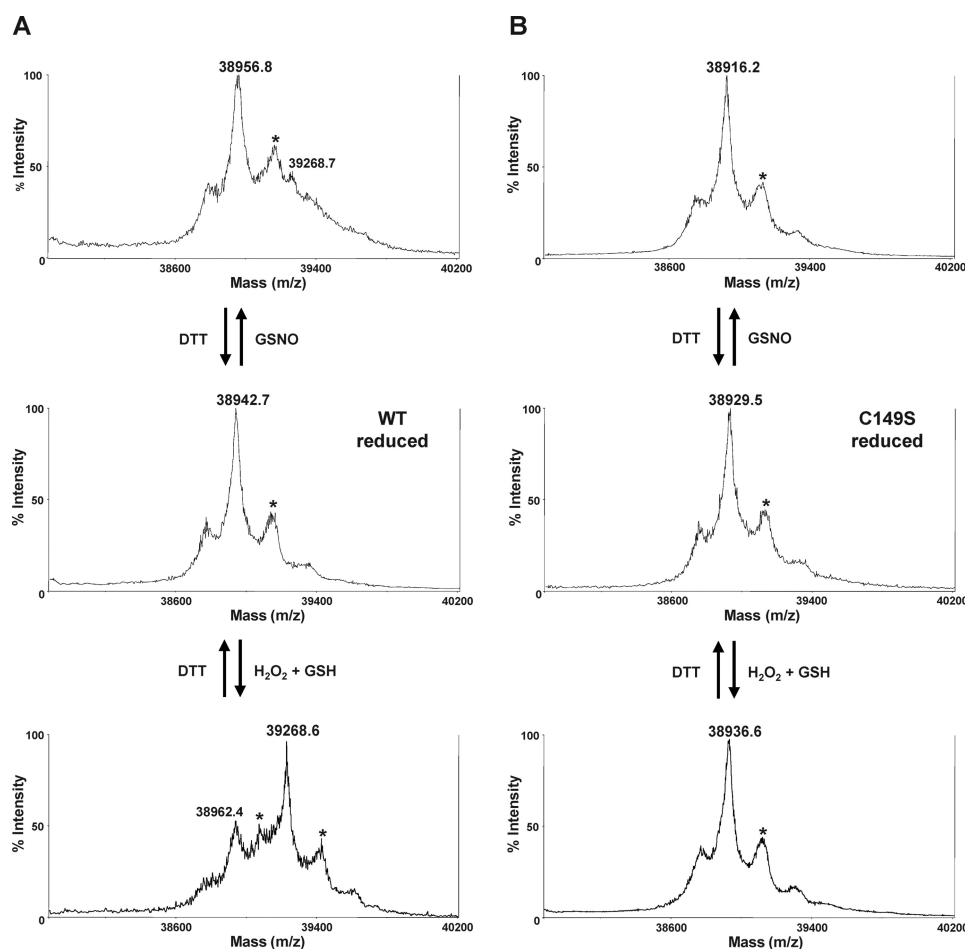


FIGURE 4. **MALDI-TOF mass spectrometry analysis.** Mass spectra of wild-type GAPC1 (WT) (A) and C149S mutant (B) treated with 1 mM GSNO for 30 min at room temperature were performed before (upper panels) and after (medium panels) a treatment in the presence of 20 mM DTT (30 min). The lower panels show the spectra of WT and C149S subjected to a typical glutathionylation treatment in the presence of 50  $\mu$ M  $H_2O_2$  and 0.5 mM GSH for 30 min at room temperature as described in Ref. 60. The differences between mass peaks of GSNO-treated GAPC1 WT and C149S mutant before (38,942.7 and 38,929.5 Da, respectively) and after DTT treatment (38,956.8 and 38,916.2 Da, respectively) are within the experimental error (0.1%) of the instrument. The peaks marked with asterisks correspond to GAPC1 (WT and C149S mutant) with a sinapinic acid adduct.

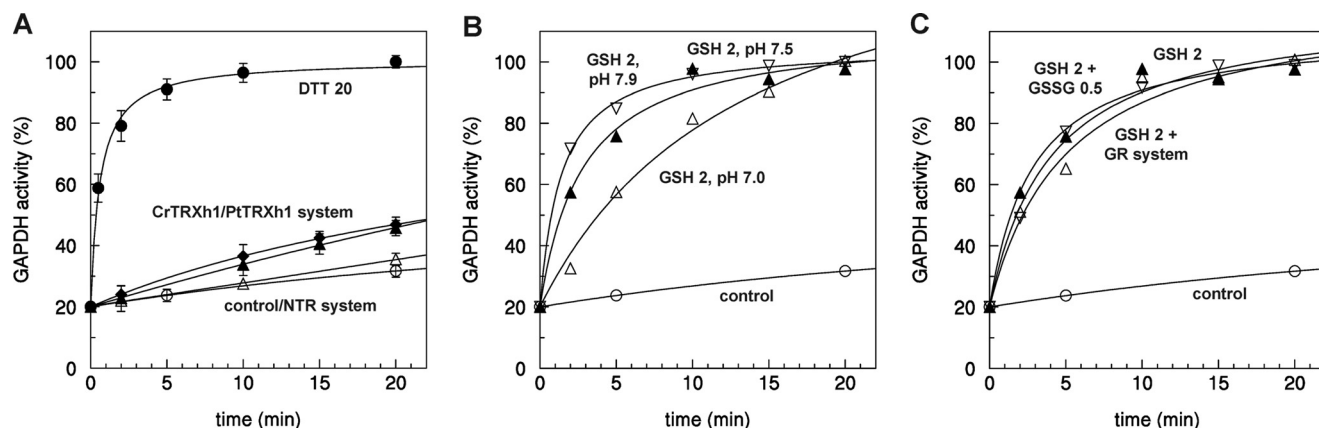


FIGURE 5. **Kinetics of reactivation of nitrosylated GAPC1.** A, nitrosylated GAPC1 was incubated with buffer as control (open circles), with 20 mM DTT (closed circles), with NTR system alone (0.2 mM NADPH and 0.22  $\mu$ M *A. thaliana* NTRb, open up-triangles), or in the presence of *Chlamydomonas* (Cr) TRXh1 (10  $\mu$ M, closed up-triangles) or poplar (Pt) TRXh1 (10  $\mu$ M, closed diamonds). Error bars represent average  $\pm$  S.D. ( $n = 3$ ). When error bars are not visible, they are within the symbol. B, nitrosylated GAPC1 was incubated with buffer as control (open circles) or in the presence of 2 mM GSH at pH 7.0 (open up-triangles), pH 7.5 (closed up-triangles), or pH 7.9 (open down-triangles). C, nitrosylated GAPC1 was incubated with buffer as control (open circles), with 2 mM GSH alone (closed up-triangles), or in the presence of 0.5 mM GSSG (open down-triangles) or GR system (0.2 mM NADPH and 6  $\mu$ g/ml glutathione reductase; open up-triangles). For all panels, aliquots of the incubation mixtures were withdrawn at the indicated time points, and the NADH-dependent activity was determined. Data represent the mean percentage ( $n = 3$ ) of maximal control activity (100% corresponds to the mean maximal reactivation measured after treatment with 20 mM DTT). Except for A, S.D. was omitted for the sake of clarity.



(NTR system). The NTR reducing system alone (Fig. 5A) or in the presence of poplar TRXh2, TRXh3, or TRXh5 (data not shown) had no effect on GAPC1 activity. A very slow reactivation could be observed with the NTR system in the presence of TRXh1 from either poplar or *Chlamydomonas* (Fig. 5A). By contrast, treatment of GAPC1-SNO with a physiological concentration of GSH (2 mM) allowed a rapid and complete recovery of GAPDH activity comparable with the reactivation kinetics observed with DTT (Fig. 5B). The efficiency of GSH-mediated reactivation decreased with decreasing pH values between 8 and 7. This result is consistent with the estimated  $pK_a$  value of GSH at 8.7 (75) and indicates that the thiolate form of GSH is involved in the reaction. The full reactivation by GSH of nitrosylated GAPC1 (Fig. 5B) clearly indicates that the GSH thiolate (more abundant at pH 7.9 than 7.0) attacks the N nucleus (and not the S nucleus) of the *S*-nitrosocysteine. As a result, the GAPC1 cysteine is reduced, and enzyme activity is restored, whereas GSNO is released. Otherwise, if the GSH thiolate attacked the S nucleus of the nitrosocysteine, GAPC1 would be consequently glutathionylated and, as such, would be insensitive to GSH reactivation, as demonstrated previously (60). Hence, full reactivation by GSH of nitrosylated GAPC1

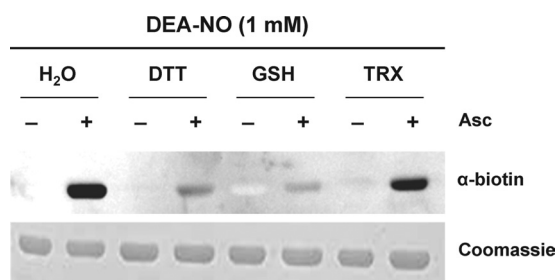
can only be explained by GSH directly removing NO from the catalytic Cys-149.

GAPC1 reactivation with 2 mM GSH was not influenced by the addition of glutathione reductase and NADPH (which keeps glutathione fully reduced during the incubation) or, on the contrary, by GSSG (Fig. 5C). These results suggest that GSH-mediated denitrosylation may not be influenced by the presence of GSSG.

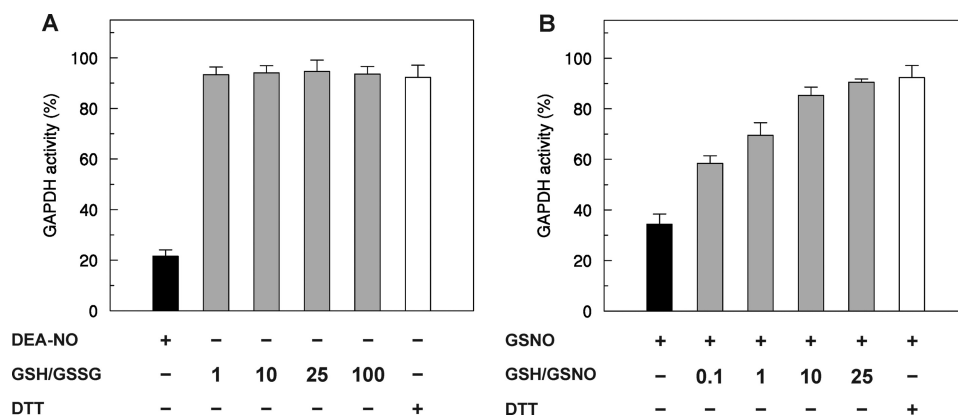
The denitrosylation of GAPC1-SNO by TRX and GSH was also analyzed with the BST assay (Fig. 6). A strong nitrosylation signal was detected for untreated GAPC1-SNO, although only a faint signal remained after treatment of the nitrosylated protein with DTT or GSH. By contrast, the signal was only slightly decreased with the NTR system supplemented with *Chlamydomonas* TRXh1, consistently with activity assays.

All of these results indicate that plant cytoplasmic TRXs are not efficient catalysts of GAPC1 denitrosylation. In contrast, GSH induces rapid denitrosylation of GAPC1 under physiologically relevant concentrations and redox states of the glutathione pool.

**GSH-dependent Denitrosylation Is Linked to the [GSH]/[GSNO] Ratio and Independent of the [GSH]/[GSSG] Ratio**—To determine the dependence of GSH-mediated denitrosylation of GAPC1 on the glutathione redox state, GAPC1-SNO was incubated in the presence of different [GSH]/[GSSG] ratios using physiological glutathione concentrations (Fig. 7A). GAPC1 is a protein found within the nucleocytoplasmic compartment, which constitutes a reducing environment where the [GSH]/[GSSG] ratio is maintained in the 10:1 to 100:1 range in diverse organisms. The most oxidizing environment is generally found in the endoplasmic reticulum where the ratio is estimated to be close to 1:1. *In vitro*, the activity of GAPC1-SNO was efficiently and fully recovered in the presence of GSH under all conditions, even when the [GSH]/[GSSG] ratio was set to 1:1, a very unlikely redox state of the glutathione pool in the nucleocytoplasmic compartment, even under artificial oxidative stress (76–78). These results indicate that GSH-mediated GAPC1 denitrosylation is totally independent of the [GSH]/[GSSG] ratio. This conclusion is consistent with the fact



**FIGURE 6. Denitrosylation of GAPC1 in the presence of reducing systems.** GAPC1 (25  $\mu$ M) was treated with 1 mM DEA-NO (30 min) and desalted on NAP-5 columns equilibrated with 50 mM Tris-HCl, 1 mM EDTA. Subsequently, the samples were incubated for 30 min in the presence of buffer, 20 mM DTT, 2 mM GSH, or TRX system (0.2 mM NADPH, 0.22  $\mu$ M *A. thaliana* NTRb, and 10  $\mu$ M *Chlamydomonas* TRXh1) and subjected to the biotin switch assay. Biotinylated proteins were visualized using anti-biotin Western blots as described under "Experimental Procedures." The Coomassie Brilliant Blue staining of the gel shows equal loading in each lane. Asc, ascorbate.



**FIGURE 7. Effect of the [GSH]/[GSSG] and [GSH]/[GSNO] ratios on the reactivation of GAPC1-SNO.** A, nitrosylated GAPC1 (black bar) was incubated in the presence of 2 mM GSH supplemented with variable amounts of GSSG (0.02, 0.08, 0.2, and 2 mM) (gray bars). After 10 min of incubation, an aliquot of the samples was withdrawn for the assay of enzyme activity. B, reduced GAPC1 was treated with 2 mM GSNO (black bar), and after 30 min of incubation, various amounts of GSH were added to obtain the indicated [GSH]/[GSNO] ratios (gray bars). After 10 min of incubation, an aliquot of the samples was withdrawn for the assay of enzyme activity. For both panels, the reactivations in the presence of 20 mM DTT are shown (white bars). Data are represented as mean percentage  $\pm$  S.D. ( $n = 3$ ) of the initial activity of reduced GAPC1 measured before the nitrosylation treatment.

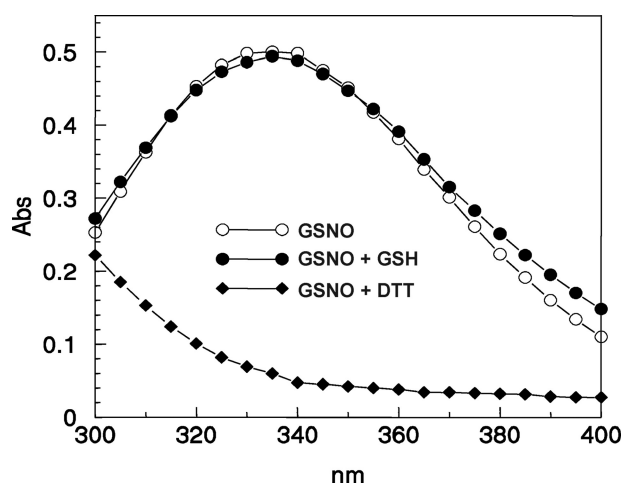


FIGURE 8. **Stability of GSNO in the presence of DTT or GSH.** UV-visible spectra (300–400 nm) were recorded after 30 min of incubation in Tris-HCl (pH 7.5), 1 mM EDTA buffer of 0.54 mM GSNO alone (open circles) or in the presence of 2 mM GSH (closed circles) or 10 mM DTT (closed diamonds).

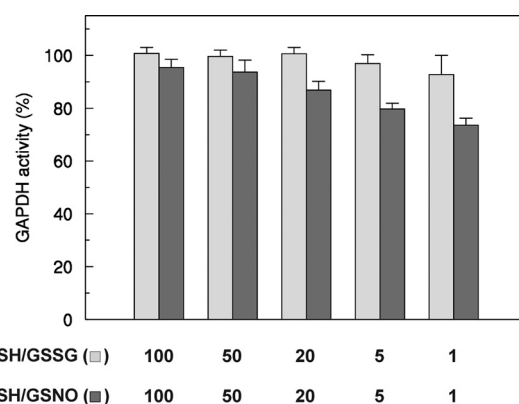


FIGURE 9. **Redox titrations of GAPC1 in the presence of various [GSH]/[GSNO] or [GSH]/[GSSG] ratios.** Reduced GAPC1 was incubated in the presence of varying [GSH]/[GSNO] (light gray bars) or [GSH]/[GSSG] (dark gray bars) ratios at a total concentration of 2 mM. After equilibrium in the redox buffers was reached (30 min), the remaining NADH-dependent activity was measured. Data are represented as mean percentage  $\pm$  S.D. ( $n = 3$ ) of the initial activity of reduced GAPC1 measured before the treatments.

that GSSG is not involved in the GSH-mediated denitrosylation reaction that most probably yields GAPC1-SH and GSNO. By contrast, the recovery of GAPC1-SNO activity increased with increasing [GSH]/[GSNO] ratios (Fig. 7B). Although GSNO (S atom) may react with GSH to release GSSG- and NO-related species, this reaction is very slow (second order rate constant of  $0.01 \text{ M}^{-1} \text{ s}^{-1}$  at  $37^\circ \text{C}$ ) (79). Consistently, we observed no significant decrease of the typical absorbance of GSNO at 335 nm in the presence of GSH, although the absorbance rapidly decreased in the presence of DTT (Fig. 8). However, GSH may also react with the N atom of GSNO, but in this case the products of the reaction (GSNO and GSH) would correspond to the substrates (GSH and GSNO), thus with no effects on [GSH]/[GSNO] ratios. Therefore, we conclude that GSH-dependent denitrosylation of GAPC1-SNO is truly correlated with the [GSH]/[GSNO] ratio. Little is known about GSNO levels and compartmentalization in plant or animal tissues. In a recent study on pepper plants, GSH/GSNO ratios were found in the same order as GSH/GSSG ratios (*i.e.*  $10^2$ ) in whole organs (roots, stem, leaves; see Ref. 40).

The effects of different [GSH]/[GSSG] and [GSH]/[GSNO] ratios on reduced GAPC1 were also analyzed (Fig. 9). Incubation with the physiological [GSH]/[GSSG] ratio in the 100:1 to 1:1 range had no significant effect on GAPDH activity, although the enzyme was progressively inhibited with decreasing [GSH]/[GSNO] ratios. Overall, these results suggest that GSSG-mediated glutathionylation of GAPC1 does not occur at physiological [GSH]/[GSSG] ratios, although the extent of GAPC1 nitrosylation may be influenced by [GSH]/[GSNO] ratios in the same range (Fig. 9).

## DISCUSSION

This study confirmed that *Arabidopsis* GAPC1 can be reversibly inactivated by nitrosylation of its catalytic Cys-149. This modification occurs by reaction of reduced GAPC1 (GAPC1-S<sup>-</sup>) with NO donors or through GSNO-mediated trans-nitrosylation (Fig. 10). This result is consistent with proteomic analyses and targeted studies that identified this enzyme

as a nitrosylated protein in *Arabidopsis* and other species (63, 80–83) and with an *in vitro* analysis of RPTMs controlling GAPC1 and GapC2 from *Arabidopsis* (64). In the latter study, the activity of nitrosylated GAPDH was not fully restored by reducing treatment in the presence of DTT suggesting that NO donors might also induce partial irreversible inactivation of *Arabidopsis* cytoplasmic GAPDH. We show here that NO donors fully inhibit the enzyme activity due essentially to nitrosylation of Cys-149 and that this inactivation is fully reversed by DTT. These biochemical properties of *Arabidopsis* cytoplasmic GAPDH are similar to those reported for mammalian GAPDH. Therefore, these results open the possibility that *Arabidopsis* GAPC1 has the ability to participate in nonglycolytic functions, as shown previously for its mammalian counterpart that participates in different regulatory and signaling functions triggered by RPTM of its catalytic cysteine and subsequent nuclear relocalization. Different plant GAPDH isoforms, including *Arabidopsis* GAPC1, may localize in the nucleus under certain conditions (64, 83–85). Although the function of GAPC1 in the nucleus is still unclear, nuclear relocalization is stimulated by stress conditions that also induce RPTMs of active site GAPDH cysteine (83, 85). Therefore, also in plants, nuclear GAPDH is likely to play completely different roles than catalyzing a glycolytic reaction.

GSNO was found to induce both nitrosylation and glutathionylation of GAPC1, although nitrosylation was largely prominent. Mammalian GAPDH was also shown to undergo both nitrosylation and glutathionylation in the presence of GSNO and to be preferentially nitrosylated (37, 86). In addition to GAPDH, several enzymes have been reported to undergo both modifications upon GSNO treatment. Although most enzymes are preferentially nitrosylated by GSNO, several enzymes such as creatine kinase (87), cytoplasmic branched chain amino-transferase (88), or papain (37) are mostly glutathionylated in the presence of this compound. Chloroplastic GRXS12 is even exclusively glutathionylated in the presence of GSNO (38, 61). The structural determinants accounting for these preferences remain unclear but likely rely on the local environment of the target cysteine, its influence on the interaction with GSNO, and

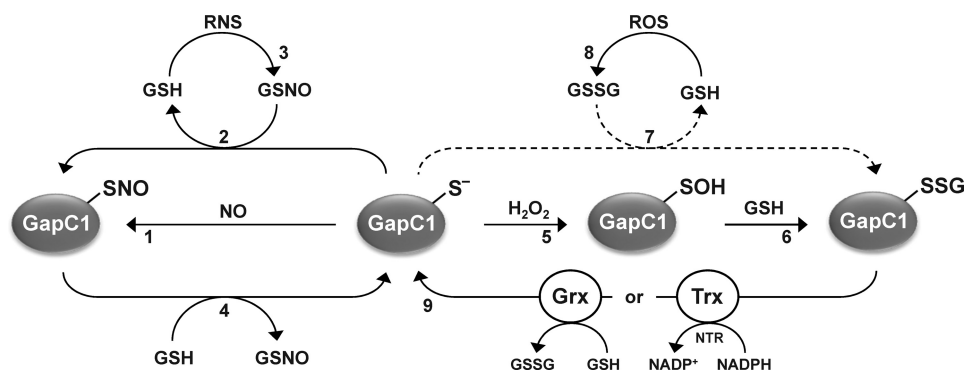


FIGURE 10. **Reactions leading to (de)nitrosylation and (de)glutathionylation of AtGAPC1.** Reduced AtGAPC1 ( $\text{GAPC1-S}^-$ ) undergoes S-nitrosylation in the presence of NO donors (1) or trans-nitrosylation by GSNO with concomitant release of GSH (2). GSNO can be generated by the reaction of GSH with RNS (3). Denitrosylation of nitrosylated GAPC1 ( $\text{GAPC1-SNO}$ ) is not catalyzed by plant cytoplasmic TRXs, but it is efficiently catalyzed by GSH with formation of GSNO (4), hence controlled by the  $[\text{GSH}]/[\text{GSNO}]$  ratio. AtGAPC1 can undergo primary oxidation to sulfenic acid ( $\text{GAPC1-SOH}$ ) in the presence of  $\text{H}_2\text{O}_2$  (5) that can subsequently react with GSH leading to the glutathionylated form ( $\text{GAPC1-SOG}$ ) (6). In principle, GAPC1 glutathionylation may also be performed by GSSG with release of GSH (7), but this reaction does not occur under physiological  $[\text{GSH}]/[\text{GSSG}]$  ratios (dashed arrow). GSSG can be formed by the reaction of GSH with ROS (8). Deglutathionylation of  $\text{GAPC1-SOG}$  can be catalyzed by either TRX system (i.e. TRX plus NADPH and NADPH:thioredoxin reductase (NTR)) or GRX system (GRX plus GSH) (9). Therefore, glutathionylation of GAPC1 is indirectly controlled by either  $[\text{NADPH}]/[\text{NADP}^+]$  and/or  $[\text{GSH}]/[\text{GSSG}]$  ratios.

on the relative stabilities of the nitrosothiol bond and the glutathionylated cysteine. That trans-nitrosylation is the preferred reaction between GAPC1 and GSNO is also consistent with the fact that GSH can denitrosylate GAPC1 with total recovery of enzyme activity, i.e. without glutathionylating GAPC1. Indeed, denitrosylation by GSH is the reverse reaction of nitrosylation by GSNO (Fig. 10), and it makes sense that both reactions may occur in the same local environment (i.e. the active site of GAPC1).

Nitrosylation and glutathionylation are two types of RPTMs that in the case of GAPC1 appear to be alternative and differently regulated. NO or GSNO induce nitrosylation of catalytic Cys-149, but nitrosylation of GAPC1 is not an intermediate step toward glutathionylation, because denitrosylation by GSH leaves the protein reduced (Fig. 10). However, sulfenation of Cys-149 by  $\text{H}_2\text{O}_2$  is a mandatory step of GSH-dependent glutathionylation, a modification that requires GRX or TRX systems for being reverted (Fig. 10) (60). Therefore, in the case of GAPC1, nitrosylation and glutathionylation are not easily interchangeable and may well play different roles *in vivo*; the former is induced by RNS and directly controlled by the  $[\text{GSH}]/[\text{GSNO}]$  ratio, and the latter is induced by ROS (e.g.  $\text{H}_2\text{O}_2$ ) and indirectly controlled by  $[\text{GSH}]/[\text{GSSG}]$  and  $[\text{NADPH}]/[\text{NADP}^+]$  ratios via GRX and TRX systems, respectively (Fig. 10).

Both nitrosylation and denitrosylation of purified GAPC1 were found to be dependent on the  $[\text{GSH}]/[\text{GSNO}]$  ratio, which is therefore likely a major factor controlling the abundance of  $\text{GAPC1-SNO}$  (Fig. 10). However, considering the physiological concentrations of GSH in the millimolar range and a  $[\text{GSH}]/[\text{GSNO}]$  ratio on the order of 100:1 (as determined in whole organs of pepper plants (40)), accumulation of  $\text{GAPC1-SNO}$  would only be possible at cell locations where local concentrations of GSNO are strongly increased at the expense of GSH, such as in the vicinity of NO-producing enzymes or in sites where GSNO reductase, the major enzyme controlling the levels of GSNO *in vivo*, may be inhibited (19, 40). Indeed, it has emerged that nitrosylation exhibits a high degree of spatiotemporal precision partly due to interaction of target proteins with

sources of the NO group, including NO synthases and GSNO (12, 16).

Nitrosylation was found involved in the signaling role of GAPDH in mammals, where nitrosylated GAPDH interacts with other proteins such as the SIAH1 ubiquitin ligase and is consequently transferred to the nucleus (57). In the nucleus, GAPDH could function as a trans-nitrosylase (36), and mechanisms may exist to stabilize GAPDH nitrosothiol under the highly reducing conditions of the nucleocytoplasmic environment. Animal GAPDH was indeed found resistant to GSH-mediated denitrosylation *in vivo* or in total extracts (48). A possible mechanism of protection could be promoted by interaction of nitrosylated GAPDH with other proteins. The binding of partner proteins could shield access to the catalytic cysteine or could induce a conformational change of GAPDH resulting in the protection of the nitrosocysteine from GSH. In the former case, denitrosylation of GAPDH would depend on the mechanisms controlling the stability of the complex between GAPDH-SNO and other proteins. It is even possible that TRX plays a role in these reactions, a hypothesis that would fit with previous observations reporting GSH-dependent denitrosylation of GAPDH in conditions where the complex is not formed and TRX-dependent denitrosylation when the complex is present (37, 53, 89).

We have clearly shown here that plant cytoplasmic TRXs are not efficient catalysts of GAPC1 denitrosylation, whereas GSH is an excellent denitrosylating agent. This indicates that GAPC1 is not a good model enzyme for the analysis of TRX-dependent denitrosylation in plants but does not necessarily preclude a role of plant TRXs as denitrosylases whose targets remain to be identified. Further studies will be required to determine the *in vivo* and *in planta* stability of nitrosylated GAPDH in *Arabidopsis*, but it should be noted that stability of the nitrosocysteine is not necessarily an essential requisite for a role of GAPC1 in signaling. In fact, nitrosylation might trigger nuclear relocation of GAPC1 (e.g. via partner recruitment), and the role played by GAPC1 in the nucleus might be independent from its nitrosylated state. For instance, both animal and plant GAPDHs display a secondary activity of uracil glyco-



ylase, an activity that prevents mutagenesis by eliminating uracil from DNA molecules (90, 91).

*Arabidopsis* GAPDH isoforms are several, and they are controlled by different types of RPTMs as follows: intramolecular disulfide bond controlled by ferredoxin-dependent TRX for A<sub>2</sub>B<sub>2</sub>-GAPDH (92, 93) and A<sub>4</sub>-GAPDH complexed with the regulatory protein CP12 (94); glutathionylation controlled by either only GRX or both TRX and GRX for A<sub>4</sub>-GAPDH and GapC, respectively (60–62); and nitrosylation controlled by GSH and GSNO for GAPC (this study). The possibility to alter GAPDH function by different types of RPTM and the fact that these RPTMs are controlled by distinct redox couples (reduced/oxidized ferredoxin for A<sub>2</sub>B<sub>2</sub>-GAPDH and CP12 disulfides; [NADPH]/[NADP<sup>+</sup>] and/or [GSH]/[GSSG] for glutathionylation; [GSH]/[GSNO] for nitrosylation) likely contributes to the functional flexibility of GAPDH allowing this enzyme of primary carbon metabolism to participate in diverse cellular processes and pathways that remain to be established in plants. GAPDH is probably one example among hundreds of moonlighting proteins that remain to be identified and characterized.

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