ANTIOXIDANTS & REDOX SIGNALING
Volume 7, Numbers 7 & 8, 2005

Forum Original Research Communication

S-Nitrosation versus *S*-Glutathionylation of Protein Sulfhydryl Groups by *S*-Nitrosoglutathione

DANIELA GIUSTARINI,[1] ALDO MILZANI,[2] GIANCARLO ALDINI,[3] MARINA CARINI,[3] RANIERI ROSSI,[1] and ISABELLA DALLE-DONNE[2]

ABSTRACT

***S*-Nitrosation of protein sulfhydryl groups is an established response to oxidative/nitrosative stress. The transient nature and reversibility of *S*-nitrosation, as well as its specificity, render this posttranslational modification an attractive mechanism of regulation of protein function and signal transduction, in analogy to *S*-glutathionylation. Several feasible mechanisms for protein *S*-nitrosation have been proposed, including transnitrosation by *S*-nitrosothiols, such as *S*-nitrosoglutathione (GSNO), where the nitrosonium moiety is directly transferred from one thiol to another. The reaction between GSNO and protein sulfhydryls can also produce a mixed disulfide by *S*-glutathionylation, which involves the nucleophilic attack of the sulfur of GSNO by the protein thiolate anion. In this study, we have investigated the possible occurrence of *S*-glutathionylation during reaction of GSNO with papain, creatine phosphokinase, glyceraldehyde-3-phosphate dehydrogenase, alcohol dehydrogenase, bovine serum albumin, and actin. Our results show that papain, creatine phosphokinase, and glyceraldehyde-3-phosphate dehydrogenase were significantly both *S*-nitrosated and *S*-glutathionylated by GSNO, whereas alcohol dehydrogenase, bovine serum albumin, and actin appeared nearly only *S*-nitrosated. The susceptibility of the modified proteins to denitrosation and deglutathionylation by reduced glutathione was also investigated.** *Antioxid. Redox Signal.* 7, 930–939.

INTRODUCTION

A WIDE RANGE OF PROTEIN MODIFICATIONS induced by reactive oxygen and nitrogen species, including formation of *S*-nitrosocysteine, cysteine sulfenic, sulfinic, and sulfonic acid, intermolecular, intramolecular, and mixed disulfides, carbonylation, and tyrosine nitration, have been identified (9, 12, 45).

The formation of the disulfide bond between protein thiol groups (PSH) and cysteine, homocysteine, and reduced glutathione (GSH), globally referred to as *S*-thiolation, generates mixed (protein/nonprotein) disulfides, altogether described as *S*-thiolated proteins. As GSH is the low-molecular-weight thiol with the highest concentration (0.5–10 m*M*) in mammalian cells, most studies focus on *S*-glutathionylation and *S*-glutathionylated proteins (PSSG) (10, 11, 17, 26, 49). This modification may have a dual role of protection from irreversible oxidation of PSH and modulation of protein function (redox regulation), so serving for cell signaling. However, if the modified cysteine is functionally critical, *S*-glutathionylation will also render the protein inactive and eventually compromise cellular functions (3, 4, 7, 13, 14, 35). Several mechanisms have been proposed for *S*-glutathionylation: reaction of GSH with partially oxidized reactive PSH (thiyl radicals or sulfenic acids), thiol–disulfide exchange between PSH and glutathione disulfide (GSSG), oxidation of GSH to the sulfenic acid (GSOH) that reacts with a PSH to form a mixed disulfide, nucleophilic attack of a protein thiolate on low-molecular-weight *S*-nitrosothiols (RSNO), and *S*-nitrosation of PSH followed by reaction with GSH to yield mixed disulfides (26).

Protein *S*-thiolation by low-molecular-weight RSNO has been extensively reported (24, 27, 32, 34, 36, 50). Indeed, *S*-

[1]Department of Neuroscience, Pharmacology Unit, University of Siena, Siena, Italy.
[2]Department of Biology, University of Milan, Milan, Italy.
[3]Istituto Chimico Farmaceutico Tossicologico, University of Milan, Milan, Italy.

thiolation is considered to be one of the mechanisms of action of RSNO, the others being nitric oxide (NO) release, transnitrosation, and, possibly, direct action (23).

RSNO are metabolites of NO that have been detected in both intra- and extracellular spaces. Apart from low-molecular-weight RSNO, such as *S*-nitrosoglutathione (GSNO), most (95%) RSNO are associated with proteins. *S*-nitrosated proteins (PSNO) so far identified include signaling molecules, enzymes, proteases, receptors, channels, G proteins, transcription factors, and extracellular matrix proteins (33, 47). Both protein and nonprotein RSNO facilitate the transport of NO *in vivo*, conferring NO properties on the carrier, while mitigating against its toxic effects (23). *S*-Nitrosation of proteins is an established response to oxidative/nitrosative stress and is emerging as a redox-sensitive posttranslational modification that is a key mechanism underlying many of the physiological effects of NO. *S*-Nitrosation has been shown to alter the function (inactivating or indeed activating) of a broad spectrum of proteins and has been suggested as a regulatory modification in cell signaling pathways, including the apoptotic process (5, 19, 20, 26, 30, 33, 41, 47).

Protein *S*-nitrosation can result from transnitrosation reactions, in which an NO^+ (nitrosonium ion) group is directly transferred from a PSNO or from a low-molecular-weight RSNO, such as GSNO, to another protein thiol (33). *S*-Nitrosation also results from binding of NO^+ or an equivalent, such as NO_2 or N_2O_3, to sulfhydryl groups or binding of $^{\bullet}NO$ to thiyl radicals (20).

Currently, *S*-nitrosation and *S*-glutathionylation are being investigated as mechanisms of redox- and NO-mediated signal transduction, as well as cellular responses to oxidative and/or nitrosative stress. Interrelations among *S*-glutathionylation, thiol oxidation, and *S*-nitrosation leading to the formation of mixed disulfides between PSH and GSH and/or PSNO may serve to translate oxidative and nitrosative stimuli into a functional response at various levels of cellular signaling (26). However, there is still uncertainty regarding how proteins are *S*-nitrosated and *S*-glutathionylated; moreover, some possible methodological flaws occurring in their detection have hampered the identification of their precise *in vivo* role (16, 40). GSNO has attracted increasing interest in recent years for its dual ability to *S*-nitrosate and *S*-glutathionylate proteins (24, 26, 27, 36, 48). The present study reports on the occurrence of *S*-glutathionylation versus *S*-nitrosation in papain, creatine phosphokinase (CPK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), alcohol dehydrogenase (ADH), bovine serum albumin (BSA), and actin upon incubation with GSNO to carry out *S*-nitrosation under physiological conditions. The reverse reactions of deglutathionylation and denitrosation have also been evaluated.

MATERIALS AND METHODS

Materials

HPLC Sephasil C18 and gel filtration PD10 columns were purchased from Pharmacia (Uppsala, Sweden). Monobromobimane was obtained from Calbiochem (La Jolla, CA, U.S.A.) and HPLC grade reagents from BDH (Poole, U.K.). Papain, BSA, ADH from *Saccharomyces cerevisiae*, CPK, and GAPDH from rabbit muscle were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). All other reagents were of analytical grade from Sigma–Aldrich Chemie GmbH.

Protein preparation

Rabbit skeletal muscle actin was prepared in 2 m*M* Tris-HCl, pH 7.5, 0.5 m*M* dithioerythritol (DTE), 0.2 m*M* ATP, 0.2 m*M* $CaCl_2$, 1.5 m*M* NaN_3 (buffer G) (8). Prior to use, actin was extensively dialyzed against buffer G without DTE.

Papain was dissolved in 50 m*M* phosphate buffer, pH 6.2, incubated with 1 m*M* dithiothreitol for 30 min, and passed through PD10 columns before utilization. BSA, ADH, and CPK were dissolved in 100 m*M* phosphate buffer, pH 7.4. GAPDH was dissolved in 50 m*M* Tris-HCl, pH 7.5.

GSNO stock solution

Fresh GSNO solutions were prepared in MilliQ water just before use by mixing equimolar concentrations of acidified GSH (0.2 *M* in 0.75 *M* HCl) and sodium nitrite, in the presence of 50 μ*M* diethylene triaminepentaacetic acid (DTPA) to limit generation of GSNO breakdown products, such as glutathione disulfide *S*-oxide [GS(O)SG], which is a highly reactive molecule that can serve as a potent protein *S*-glutathionylating agent (48). After 2 min, the mixture was neutralized with 1 m*M* Tris and kept on ice, always protected from light. The GSNO concentration was determined as described below. All the GSNO solutions contained <4% GSSG, as checked by HPLC (15).

Determination of GSNO degradation products by electrospray ionization mass spectrometry (ESI-MS) analyses

ESI-MS analyses were performed on a Thermo Finnigan LCQ Advantage Ion Trap Mass Spectrometer (Thermo Finnigan Italia, Milan, Italy), operating in the following conditions: capillary temperature, 200°C; ionization voltage, 5 kV; capillary voltage, 3.44 V. The flow rate of the nebulizer gas (nitrogen) was 0.5 L/min. Samples were diluted to 100 μ*M* with H_2O:CH_3CN:HCOOH (70/30/0.1, by volume) and then introduced into the mass spectrometer at a flow rate of 25 μl min^{-1}. Spectra were acquired in positive-ion modes, with a scan range from *m/z* 100 to 700 (scan rate 0.5 scan/s) and analyzed by Xcalibur software.

Reactivity of proteins with GSNO or GSSG

Proteins (50 μ*M*) were treated with 1 m*M* GSNO or GSSG at 25°C; 0.4-ml aliquots were periodically removed from the incubation mixture and passed through PD10 columns to remove excess low-molecular-weight reagents; the PSNO or PSSG concentration was determined in the eluate as described below.

In denitrosation and dethiolation experiments, proteins pretreated with 1 m*M* GSNO for 2 h or 1 m*M* GSSG for 10 h and reisolated on PD10 columns were then treated with 0.5 m*M* GSH at 25°C. At various time intervals, 0.4-ml aliquots were passed through PD10 columns to remove excess GSH, and assayed for PSNO or PSSG concentration.

The absence of contaminating GSH, GSSG, GSNO, and nitrite was verified in every eluate by both HPLC (GSH and GSSG) (15) and the Griess reaction (44) (GSNO and nitrite). All buffers contained 50 μ*M* DTPA to limit generation of RSNO breakdown products.

Determination of RSNO

The GSNO and PSNO concentrations were determined spectrophotometrically at 540 nm by the Griess reaction (44). Quantification was performed by reference to a standard curve obtained using authentic nitrite.

Determination of protein S*-glutathionylation*

At different time intervals, proteins were isolated by low-molecular-weight reagents by gel filtration on PD10 columns. Protein *S*-glutathionylation was measured by reversed-phase HPLC (10, 15). Derivatized thiols were analyzed by fluorescence detection (excitation, 380 nm; emission, 480 nm) and quantified using authentic GSH similarly derivatized with monobromobimane.

RESULTS

We have examined the occurrence of competing *S*-nitrosation and *S*-glutathionylation, triggered by incubation with GSNO, in different proteins known for having one or more free cysteines susceptible to oxidative/nitrosative modifications. Papain has a critical cysteine in the active site, which is thought to be catalytically active, together with His^{159}, as a thiolate-imidazolium ion pair (50). The cytoplasmic form of CPK has one reactive cysteine per subunit, and its oxidative modifications completely inhibit the enzyme activity (27). GAPDH is a homotetramer comprised of four identical subunits. Each subunit contains four free cysteines, two of which are located in the catalytic site: Cys^{149} interacts with a histidine to form a highly reactive thiolate group, which is required for GAPDH activity (48). ADH is a dimer containing two zinc atoms per subunit; one zinc atom is found in the catalytic site bound to Cys^{46}, His^{67}, and Cys^{174}; the second zinc, which plays a structural role, is bound to four cysteines. Actin and BSA have five and 35 Cys, respectively, but both possess only a single, highly reactive free cysteine (Cys^{374} and Cys^{34}, respectively).

The nature of protein thiol modifications following incubation with GSNO was determined at different time intervals up to 60 min (Figs. 1 and 2). All the examined proteins became *S*-nitrosated, although both the degree and the kinetics of *S*-nitrosation were different. Papain and GAPDH showed the fastest *S*-nitrosation, the maximum degree of thiol modification being reached within one or a few minutes, respectively (Fig. 1). Conversely, actin *S*-nitrosation showed the slowest progression, and failed to reach the maximum extent within 60 min (Fig. 2); this result agrees with previous data, showing that actin *S*-nitrosation by GSNO is a slow process (8). By comparing the extent of *S*-nitrosation (Figs. 1 and 2), it appeared that GAPDH showed the highest degree of *S*-nitrosation, whereas papain the lowest.

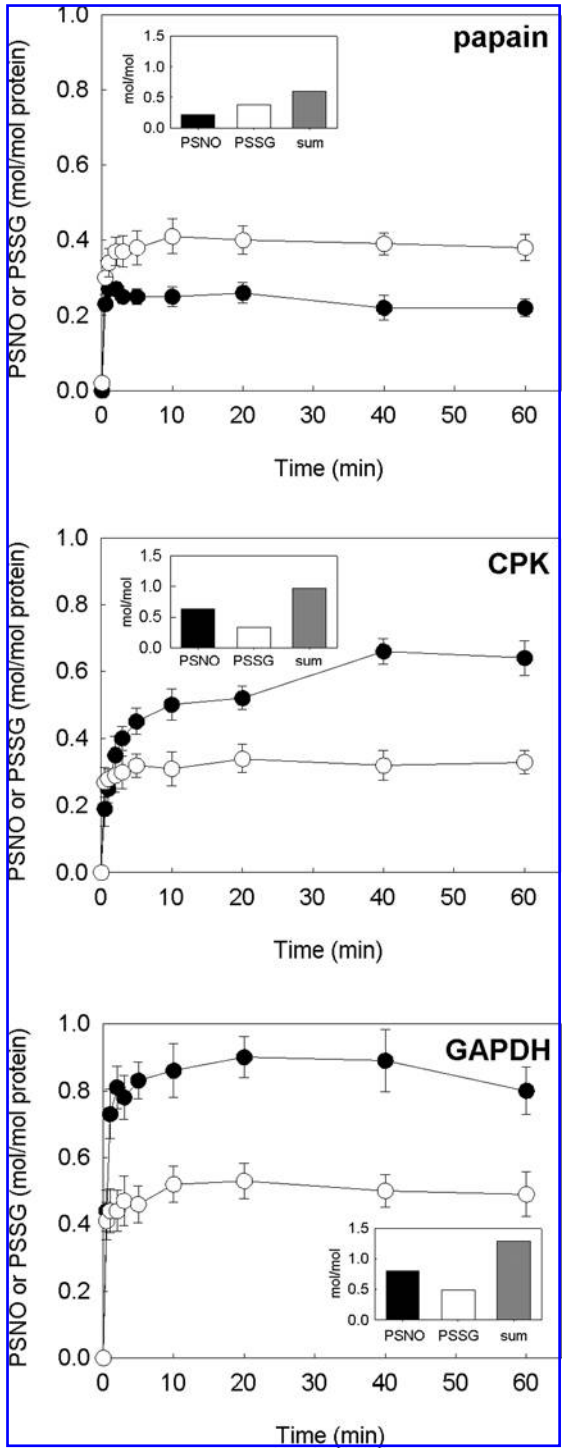

FIG. 1. *S*-Nitrosation versus *S*-glutathionylation of papain, CPK, and GAPDH induced by GSNO. Proteins (50 μ*M*) were treated with 1 m*M* GSNO. At the indicated times, sample aliquots were passed through PD10 columns, and PSNO (black circles) and PSSG (white circles) concentrations were quantified spectrophotometrically and by HPLC, respectively. (**Insets**) Concentration of PSNO (black bars), PSSG (white bars), and total modified thiols (PSNO + PSSG) (gray bars) measured after 1-h incubation with 1 m*M* GSNO. Data points represent means ± SD ($n = 5$).

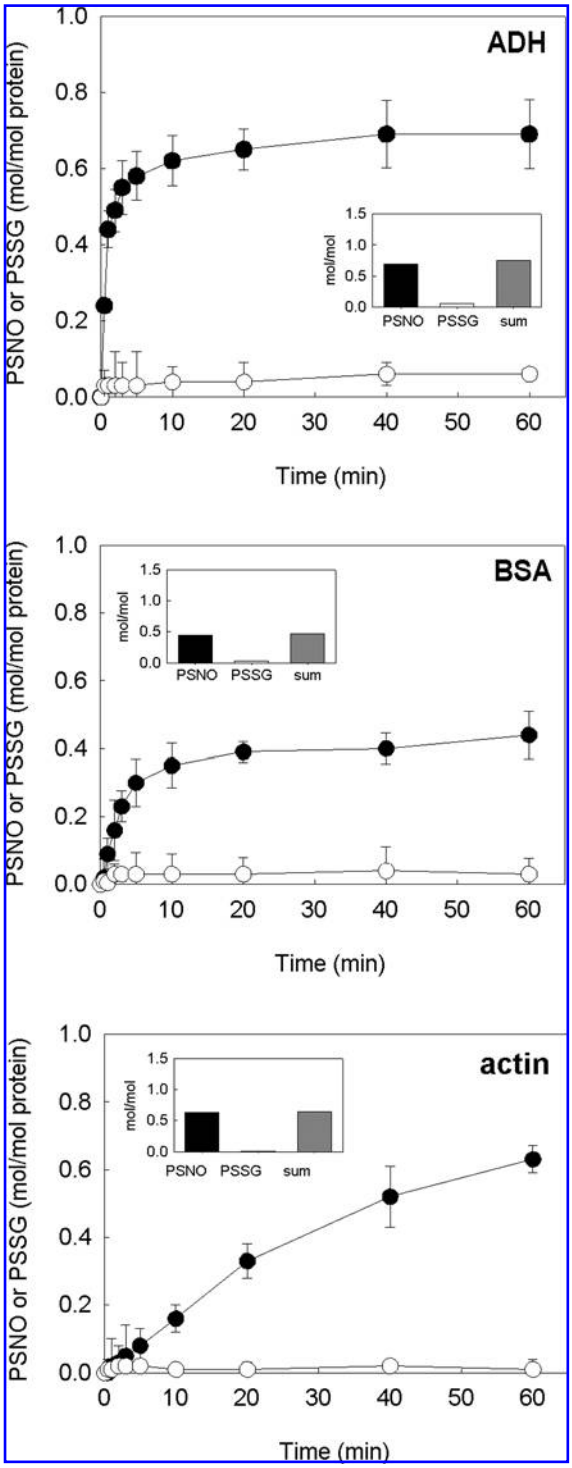

FIG. 2. *S*-nitrosation versus *S*-glutathionylation of ADH, BSA, and actin induced by GSNO. Proteins (50 μ*M*) were treated with 1 m*M* GSNO. At the indicated times, sample aliquots were passed through PD10 columns, and PSNO (black circles) and PSSG (white circles) concentrations were determined spectrophotometrically and by HPLC, respectively. (**Insets**) Concentration of PSNO (black bars), PSSG (white bars), and total modified thiols (PSNO + PSSG) (gray bars) measured after 1-h incubation with 1 m*M* GSNO. Data points represent means ± SD ($n = 5$).

Unlike *S*-nitrosation, GSNO-induced *S*-glutathionylation occurred only in papain, CPK, and GAPDH (Fig. 1), being negligible or completely absent in ADH, BSA, and actin (Fig. 2). *S*-Glutathionylation was the predominant thiol modification in papain. Insets of Figs. 1 and 2 show the degree of total thiol modification (*S*-nitrosation plus *S*-glutathionylation) after 1-h treatment with GSNO. CPK and GAPDH showed the highest molar ratio of modified thiols per protein (Fig. 1). In the case of ADH, BSA, and actin, (almost) all the modified thiols could be accounted for by protein *S*-nitrosation (Fig. 2).

Although precautions were taken to limit the breakdown of GSNO, by inclusion of the metal ion chelator DTPA, it is possible that small amounts of GSSG were formed. As GSSG is known for its ability to produce PSSG by a thiol–disulfide exchange reaction (26), we compared the kinetics of PSSG formation induced by GSSG with that induced by GSNO. Treatment with 1 m*M* GSSG for 1 h revealed that GSSG is not an effective glutathionylating agent for ADH, BSA, actin, and papain (Fig. 3). A prolonged incubation time (up to 4 h) similarly did not induce any *S*-glutathionylation in those proteins (data not shown). In contrast, GSSG induced *S*-glutathionylation of GAPDH and CPK, but the level of thiol modification at both 60 min (Fig. 3) and 4 h (data not shown) did not reach that seen upon 1-h treatment with 1 m*M* GSNO (Fig. 1), and the reaction was much slower. This indicated that the rapid *S*-glutathionylation of papain, CPK, and GAPDH observed upon incubation with GSNO (Fig. 1) does not occur, at least to a significant extent, as a result of the formation of GSSG as a GSNO breakdown product. Anyway, the formation of GSSG and other breakdown products of GSNO, like GS(O)SG, in GSNO solutions was checked by ESI-MS analysis. In Fig. 4, the spectra of a freshly prepared GSNO sample (panel A) and the same sample after 1 h (panel B) are shown. In freshly prepared GSNO, the amount of GSSG is negligible

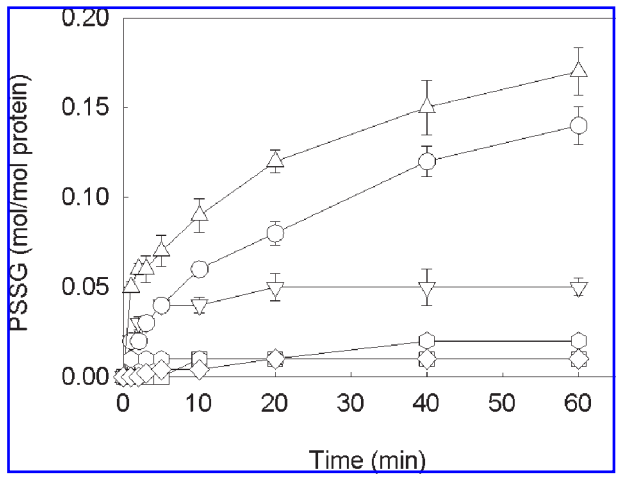

FIG. 3. Protein *S*-glutathionylation induced by GSSG. Proteins (50 μ*M*) were treated with 1 m*M* GSSG. At the indicated times, sample aliquots were passed through PD10 columns, and PSSG concentration was determined by HPLC. Upside down triangles, papain; circles, CPK; triangles, GAPDH; squares, ADH; hexagons, BSA; rhombuses, actin. Data points represent means ± SD ($n = 5$).

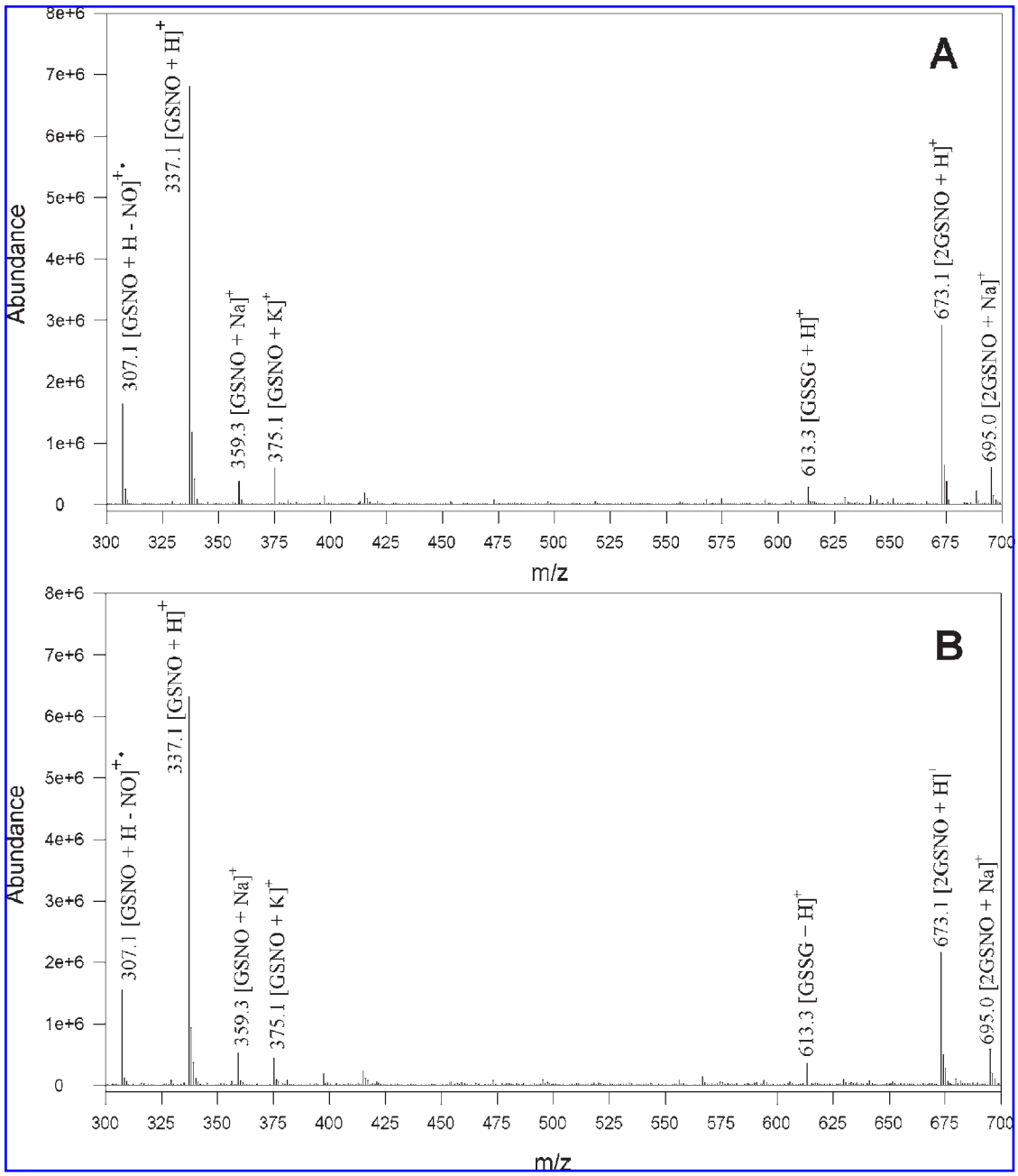

FIG. 4. ESI-MS analysis of GSNO solutions. Freshly prepared (**A**) GSNO was diluted to 100 μ*M* with a mixture of H_2O:CH_3CN:HCOOH (70/30/0.1, by volume) and infused at a flow rate of 25 μl min^{-1} into the ion trap mass spectrometer; the same solution was allowed to stand at room temperature in the dark for 1 h (**B**) and then was diluted and infused as described above.

(Fig. 4A; relative abundance <5% with respect to the base peak of GSNO) and, over the course of the experiment, it increases minimally (Fig. 4B). Moreover, there is no evidence that other breakdown products of GSNO can be formed under our experimental conditions. Thus, the possibility that *S*-glutathionylation could be due to molecules other than GSNO was ruled out.

The presence of millimolar GSH in a cellular environment could minimize *S*-nitrosation and *S*-glutathionylation reactions, although a number of reports indicate that stable, intracellular protein RSNO can exist (5, 25, 28, 31, 32, 51). The reverse reactions of denitrosation and deglutathionylation were then analyzed upon incubation of PSNO and PSSG with 0.5 m*M* GSH for different time intervals. In all the PSNO, except actin, GSH treatment induced the release of all, or nearly all, their NO content (Figs. 5 and 6). In contrast, *S*-nitrosated actin showed a slower and partial release of bound NO when exposed to excess GSH (Fig. 6). The release of bound GSH from the proteins that were also *S*-glutathionylated upon incubation with GSNO (papain, CPK, and GAPDH) was slower

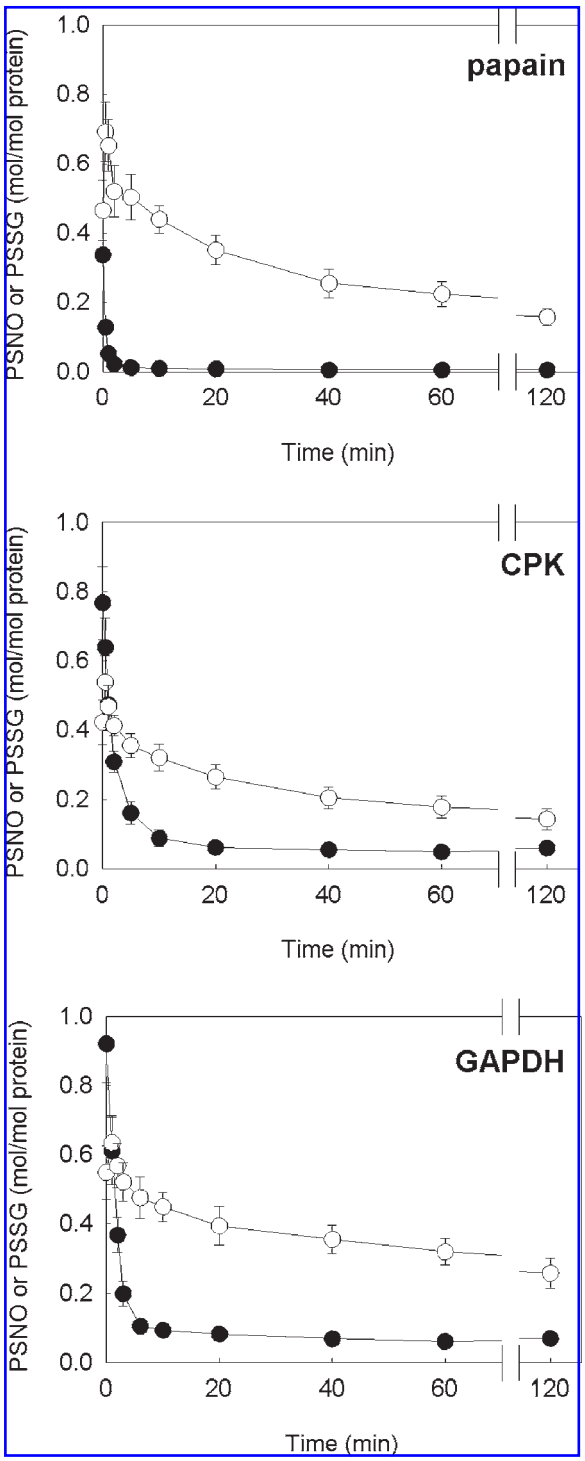

FIG. 5. Denitrosation and deglutathionylation of papain, CPK, and GAPDH. Proteins (50 μ*M*) were treated with 1 m*M* GSNO for 2 h. Samples were passed through PD10 columns, and reisolated proteins were treated with 0.5 m*M* GSH. At the indicated times, sample aliquots were passed through PD10 columns, and the levels of PSNO (black circles) and PSSG (white circles) were measured spectrophotometrically and by HPLC, respectively. Data points represent means ± SD ($n = 5$).

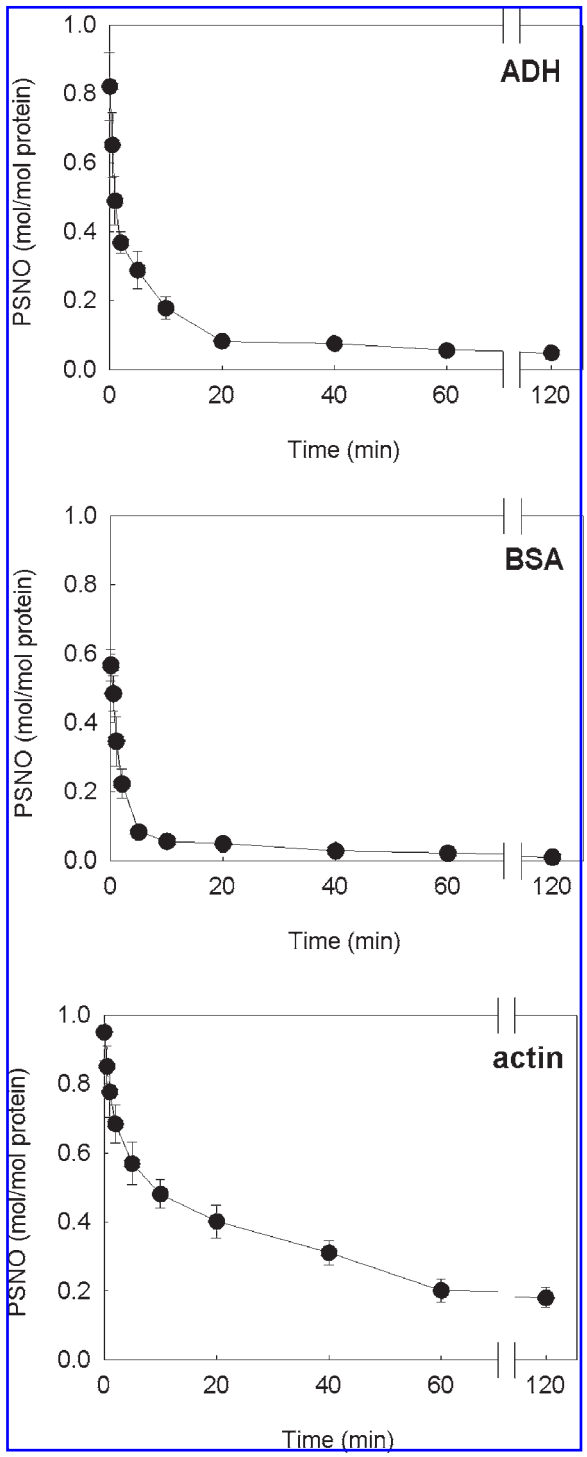

FIG. 6. Denitrosation of ADH, BSA, and actin. Proteins (50 μ*M*) were treated with 1 m*M* GSNO for 2 h. Samples were passed through PD10 columns, and reisolated proteins were treated with 0.5 m*M* GSH. At the indicated times, sample aliquots were passed through PD10 columns, and the levels of PSNO (black circles) were measured spectrophotometrically. Data points represent means ± SD ($n = 5$).

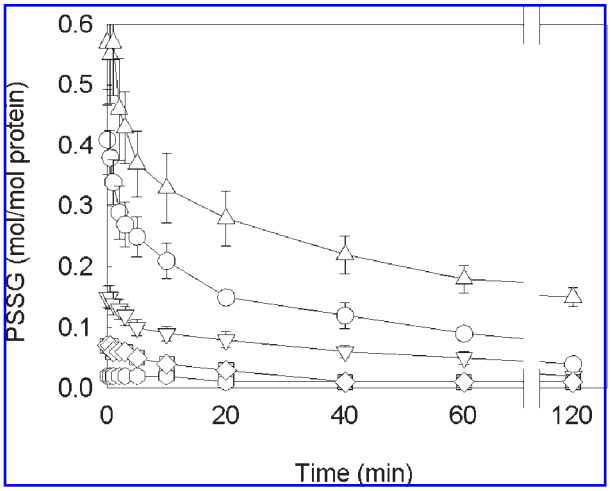

FIG. 7. Deglutathionylation of proteins pretreated with GSSG. Proteins (50 μ*M*) were treated with 1 m*M* GSSG for 10 h. Samples were passed through PD10 columns, and reisolated proteins were treated with 0.5 m*M* GSH. At the indicated times, sample aliquots were passed through PD10 columns, and PSSG concentration was determined by HPLC. Data points represent means ± SD ($n = 4$). For symbol definitions, see Fig. 3.

than denitrosation, as well as incomplete, even after 2 h from GSH addition (Fig. 5). Interestingly, a rapid, further increase in PSSG was initially observed in papain, CPK, and GAPDH, suggesting that, in some concurrently *S*-nitrosated as well as *S*-glutathionylated proteins, an initial *S*-glutathionylation may occur during the denitrosation reaction.

Finally, we evaluated the kinetics of deglutathionylation, triggered by GSH addition, in proteins treated with 1 m*M* GSSG (Fig. 7), *i.e.*, in proteins that were only *S*-glutathionylated. We did not observe the rapid increase in PSSG levels in papain, CPK, and GAPDH as seen in Fig. 5; further, deglutathionylation reactions proceeded faster than in the corresponding experiment performed on GSNO-treated proteins (Fig. 5). On the whole, these data indicate that (a) denitrosation (Figs. 5 and 6) is usually largely faster than deglutathionylation (Figs. 5 and 7), and (b) deglutathionylation of proteins presenting both *S*-nitrosated and *S*-glutathionylated thiols (Fig. 5) is slower than that of proteins that are only *S*-glutathionylated.

DISCUSSION

Proteins containing reactive sulfhydryl groups can be *S*-nitrosated and/or *S*-glutathionylated by low-molecular-weight RSNO (34, 36, 50). Although the factors governing cysteine reactivity toward nitrosating agents are not completely understood and several feasible mechanisms for protein *S*-nitrosation have been proposed (20), critical features include basic and acidic residues flanking the reactive cysteine, either in linear sequence or as a consequence of the three-dimensional organization of the protein (1, 21, 46). It has also been suggested that an important factor in determining the rates of interaction between *RSNO* and proteins is the accessibility of the protein thiolate to either the nitrogen or the sulfur of the RSNO (27).

A spate of recent observations indicates that both intracellular and extracellular (stable) PSNO can exist *in vivo* (6, 18, 32, 37, 39, 42, 47, 51), and *S*-nitrosation has been suggested to regulate, in intact cells, the function of a broad spectrum of proteins (25, 47). Important roles for *S*-nitrosation have been recently shown in vesicle-mediated insulin release (43), in protein processing associated with the neurodegeneration in Parkinson's disease, where parkin, a ubiquitin ligase, has been shown to be inhibited by *S*-nitrosation in neuronal tissue after activation of either neuronal or inducible NO synthase (6), and in the physiological regulation of nuclear factor-κB activity (42).

Protein thiol modification by transnitrosation (reaction 1) is believed to occur *in vitro* and *in vivo* (27, 38) and represents a potential mechanism whereby RSNO can posttranslationally modify PSH.

$$PSH + GSNO \rightleftarrows PSNO + GSH \quad (1)$$

The kinetics and equilibrium of this reaction are usually well described by a reversible second order process (22).

S-Nitrosation and transnitrosation of protein thiols, often leading to the alteration (either enhancement or inhibition) of enzyme/protein activity/function, have been reported for a number of purified proteins (8, 20, 27).

In recent years, increasing attention has been turned to *S*-glutathionylation as an alternative protein modification competing with *S*-nitrosation upon incubation with low-molecular-weight RSNO (24, 27, 34, 36, 50). Point charge calculations based on atom electronegativities indicate that the S-N bond of RSNO has a slight polarity with a partial negative charge on the sulfur atom and a slight positive charge on the nitrogen (27). Therefore, in transnitrosation reactions, nucleophilic attack on nitrogen (by thiolate anions) should be favored over sulfur (reaction 1). However, some evidence suggests that an attack of protein thiolates on the partially negatively charged sulfur of the S-N bond may also occur (24, 26, 34, 36). This may result not only from the reaction of a PSH with low-molecular-weight RSNO (*e.g.*, GSNO) (reaction 2), but also during denitrosation of PSNO by GSH (reaction 3) (26, 36).

$$PSH + GSNO \rightarrow PSSG + NO^- + H^+ \quad (2)$$

$$PSNO + GSH \rightarrow PSSG + NO^- \quad (3)$$

Then thiol-containing proteins may be modified by RSNO to form PSNO and/or PSSG.

All the proteins we have studied, except actin, were rapidly *S*-nitrosated via reaction 1 after treatment with GSNO (Figs. 1 and 2). Further, an extensive degree of *S*-glutathionylation was observed, by reactions 2 and 3, in papain, CPK, and GAPDH, formation following a kinetics similar to that observed for *S*-nitrosation (Fig. 1). The susceptibility of these proteins to be *S*-glutathionylated was previously suggested, but, except for CPK, without a quantitative approach (27, 34, 50).

In contrast, *S*-glutathionylation was negligible or absent in ADH, BSA, and actin (Fig. 2). This is in agreement with data

resulting from GSSG treatment, showing a lower susceptibility for ADH, BSA, and actin to be *S*-glutathionylated by GSSG (Fig. 3). With regard to this, we have recently demonstrated that alternative pathways to the thiol–disulfide exchange reaction with GSSG are needed to induce PSSG formation in actin (11).

The protein-GSH mixed disulfide production observed essentially in papain, CPK, and GAPDH can be attributed to the reaction of the sulfur atom of GSNO with the protein thiolate (reaction 2) and/or to the intermediate production of PSNO (reaction 3). The GSH released from reaction 1 may react with PSNO, thus inducing PSSG increase. The involvement of reaction 3 is also suggested from the experiment illustrated in Fig. 5, showing that treatment with GSH of simultaneously *S*-nitrosated and *S*-glutathionylated proteins leads to an initial increase in PSSG, followed by a slow decrease. Two different processes likely occur: the addition of GSH promotes protein deglutathionylation (reaction 4) but, concurrently, allows denitrosation, thus producing additional PSSG (reaction 3).

$$\text{PSSG} + \text{GSH} \rightleftarrows \text{PSH} + \text{GSSG} \qquad (4)$$

The rapid, initial increase in PSSG content measured in the experiment reported in Fig. 5 also indicates that reaction 3 is faster than reaction 4.

These results were not influenced by the presence of eventual GSSG resulting from GSNO degradation, because GSSG reacted too slowly with PSH (Fig. 3), and the kinetics of *S*-glutathionylation was not comparable to that observed upon incubation with GSNO (Figs. 1 and 2).

It is not known if a consensus motif for the propensity of a protein cysteine to undergo GSNO-induced *S*-glutathionylation similar to that described for *S*-nitrosation can exist (1, 46). However, parameters that likely take part are the cysteine pK_a and steric factors. It has been suggested that the presence of a nearby histidine could activate the cysteine in GAPDH to form a strong nucleophile (histidine-activated thiolate) in its active site, which could attack the S-NO bond of the low-molecular-weight RSNO, leading to the preferential formation of a PSSG (34). The same theory could explain the predominant *S*-glutathionylation occurring in papain in response to incubation with GSNO, given the presence of the histidine-activated thiolate group (Cys^{25}) in this cysteine protease (50), as well as the different behavior of ADH, which does not show as much susceptibility in forming PSSG as GAPDH. In fact, ADH contains one cysteine per subunit that is not activated by a histidine; the lower nucleophilicity of ADH cysteine should impede it to react with the sulfur atom in the S-NO bond. Therefore, ADH does not become *S*-glutathionylated, but only *S*-nitrosated, by GSNO in comparison with GAPDH. However, in BSA, the single Cys^{34} is located in a hydrophobic pocket adjacent to His^{39}, but, when it reacts with GSNO or other low-molecular-weight RSNO, the transnitrosation, instead of *S*-glutathionylation, takes place (27). As the thiolate anion is more reactive than the SH group, it could be hypothesized that its nucleophilic attack is faster, but not preferentially addressed toward the sulfur of GSNO rather than the nitrogen. Further investigations on the three-dimensional structure of proteins and, in particular, the identification of amino acids surrounding SH groups and charge distribution could help to explain the reasons for such a different behavior. Moreover, the relative importance of *S*-nitrosation and *S*-glutathionylation likely depends not only on the environment of the PSH, but also on the chemical nature of the low-molecular-weight RSNO, which can affect the accessibility of the protein thiolate to either the nitrogen or the sulfur of the RSNO, as shown *in vitro* for CPK, which is predominantly either *S*-thiolated or *S*-nitrosated, depending on the different species of RSNO it is exposed to (27).

Our data suggest that *S*-glutathionylation can be a quantitatively relevant process that may run in parallel to *S*-nitrosation in some proteins, and that denitrosation is a largely faster process than deglutathionylation (Fig. 5). In particular, GSNO-glutathionylated proteins seem less susceptible to deglutathionylation induced by GSH than GSSG-glutathionylated ones (Figs. 4 and 6), probably owing to concomitant reactions involving proteins that are both *S*-nitrosated and *S*-glutathionylated. Thus, in a physiological context, where probably small amounts of RSNO can be produced under particular conditions and microenvironments (51), it is possible that the NO group is exchanged among various PSH by GSH via the relatively fast reaction 1. However, when GSNO finds a suitable thiol group, also reaction 2 may occur. It should be emphasized that reaction 2 is not an equilibrium, but it is irreversible by reaction of NO^- with oxygen or other mechanisms. Furthermore, reactions 2 and 3 result in the destruction of the RSNO, thus acting as regulatory tools for the intracellular/extracellular RSNO pool in a reaction that may contribute to the GSNO-metabolizing activity of intracellular enzymes (29).

In summary, the regulatory effect that may be due to the formation of a poorly stable PSNO—which, within cells, is usually bathed in millimolar concentrations (unless present in a compartment that is not solvent-exposed) of GSH that rapidly denitrosates the protein via transnitrosation, and the resulting GSNO is possibly consumed by a putative "GSNO reductase" activity of GSH-dependent formaldehyde dehydrogenase (29)—can be reinforced by the transformation of the PSNO into a PSSG. Our data agree with the finding of stable intracellular protein RSNO, when they are likely located either in hydrophobic protein pockets inaccessible to GSH and other reducing agents or in specific cellular microenvironments (51).

S-Nitrosation and *S*-glutathionylation of PSH are recognized as physiological responses to nitrosative/oxidative stress, but how proteins are *S*-glutathionylated and *S*-nitrosated *in vivo* is not well established, although recent studies have attempted to shed light on this issue (2, 51, 52). The effect of *S*-nitrosation and *S*-glutathionylation on protein activity may be similar, and both modifications are reversible, but the resulting products, PSNO and PSSG, may possess different biological properties. For instance, transnitrosation provides a mechanism by which a thiol can be *S*-nitrosated in an appropriate cellular/physiological environment and then serve as a source of NO^+ for other thiols at distal sites. Reversible *S*-nitrosation and *S*-glutathionylation of PSH generate interest as regulatory events that potentially modify a large number of processes in response to oxidative/nitrosative stress. The role of *S*-nitrosation is much more controversial because no controlled enzymatic mechanism of RSNO for-

mation has yet been discovered. Although RSNO degradation pathways exist (29), it is not clear how they are specifically related to NO signaling processes.

Examination of nearly any metabolic or signal transduction pathway produces a number of candidate proteins that might be altered by oxidative/nitrosative modifications. Thus, the significance of the combined effects of *S*-nitrosation/denitrosation and *S*-glutathionylation/deglutathionylation on different proteins may produce cellular metabolic changes necessary for survival during short-term oxidative/nitrosative stress. In the future, it will be necessary to understand these processes by combining many individual protein modifications into a global metabolic plan in order to understand fully the importance of reversible oxidative/nitrosative protein modifications.

ACKNOWLEDGMENTS

This work was supported by Monte dei Paschi di Siena and by FIRST 2004 (Fondo Interno Ricerca Scientifica e Tecnologica), University of Milan.

ABBREVIATIONS

ADH, alcohol dehydrogenase; BSA, bovine serum albumin; CPK, creatine phosphokinase; DTE, dithioerythritol; DTPA, diethylenetriaminepentaacetic acid; ESI-MS, electrospray ionization mass spectrometry; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, reduced glutathione; GSNO, *S*-nitrosoglutathione; GS(O)SG, glutathione disulfide *S*-oxide; GSSG, glutathione disulfide; NO, nitric oxide; NO^+, nitrosonium ion; PSH, protein sulfhydryl groups; PSNO, *S*-nitrosated proteins; PSSG, *S*-glutathionylated proteins; RSNO, *S*-nitrosothiols.

REFERENCES

1. Ascenzi P, Colasanti M, Persichini T, Muolo M, Polticelli F, Venturini G, Bordo D, and Bolognesi M. Re-evaluation of amino acid sequence and structural consensus rules for cysteine-nitric oxide reactivity. *Biol Chem* 381: 623–627, 2000.
2. Bryan NS, Rassaf T, Maloney RE, Rodriguez CM, Saijo F, Rodriguez JR, and Feelisch M. Cellular targets and mechanisms of nitros(yl)ation: an insight into their nature and kinetics in vivo. *Proc Natl Acad Sci U S A* 101: 4308–4313, 2004.
3. Caplan JF, Filipenko NR, Fitzpatrick SL, and Waisman DM. Regulation of annexin A2 by reversible glutathionylation. *J Biol Chem* 279: 7740–7750, 2004.
4. Casagrande S, Bonetto V, Fratelli M, Gianazza E, Eberini I, Massignan T, Salmona M, Chang G, Holmgren A, and Ghezzi P. Glutathionylation of human thioredoxin: a possible crosstalk between the glutathione and thioredoxin systems. *Proc Natl Acad Sci U S A* 99: 9745–9749, 2002.
5. Choi YB, Tenneti L, Le DA, Ortiz J, Bai G, Chen HS, and Lipton SA. Molecular basis of NMDA receptor coupled ion channel modulation by *S*-nitrosylation. *Nat Neurosci* 3: 15–21, 2000.
6. Chung KKK, Dawson TM, Thomas B, Li X, Pletnikova O, Troncoso JC, Marsh L, and Dawson VL. *S*-Nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. *Science* 304: 1328–1331, 2004.
7. Cross JV and Templeton DJ. Oxidative stress inhibits MEKK1 by site-specific glutathionylation in the ATP binding domain. *Biochem J* 381: 675–683, 2004.
8. Dalle-Donne I, Milzani A, Giustarini D, Di Simplicio P, Colombo R, and Rossi R. *S*-NO-actin: *S*-nitrosylation kinetics and the effect on isolated vascular smooth muscle. *J Muscle Res Cell Motil* 21: 171–181, 2000.
9. Dalle-Donne I, Giustarini D, Colombo R, Rossi R, and Milzani A. Protein carbonylation in human diseases. *Trends Mol Med* 9: 169–176, 2003.
10. Dalle-Donne I, Giustarini D, Rossi R, Colombo R, and Milzani A. Reversible *S*-glutathionylation of Cys(374) regulates actin filament formation by inducing structural changes in the actin molecule. *Free Radic Biol Med* 34: 23–32, 2003.
11. Dalle-Donne I, Rossi R, Giustarini D, Colombo R, and Milzani A. Actin *S*-glutathionylation: evidence against a thiol–disulphide exchange mechanism. *Free Radic Biol Med* 35: 1185–1193, 2003.
12. Dalle-Donne I, Scaloni A, Giustarini D, Cavarra E, Tell G, Lungarella G, Colombo R, Rossi R, and Milzani A. Proteins as biomarkers of oxidative/nitrosative stress in diseases: the contribution of redox-proteomics. *Mass Spectrom Rev* 24: 55–99, 2005.
13. Eaton P, Byers HL, Leeds N, Ward MA, and Shattock MJ. Detection, quantitation, purification, and identification of cardiac proteins *S*-thiolated during ischemia and reperfusion. *J Biol Chem* 277: 9806–9811, 2002.
14. Eaton P, Wright N, Hearse DJ, and Shattock MJ. Glyceraldehyde phosphate dehydrogenase oxidation during cardiac ischemia and reperfusion. *J Mol Cell Cardiol* 34: 1549–1560, 2002.
15. Giustarini D, Dalle-Donne I, Colombo R, Petralia S, Giampaoletti S, Milzani A, and Rossi R. Protein glutathionylation in erythrocytes. *Clin Chem* 49: 327–330, 2003.
16. Giustarini D, Milzani A, Colombo R, Dalle-Donne I, and Rossi R. Nitric oxide, *S*-nitrosothiols and hemoglobin: is methodology the key? *Trends Pharmacol Sci* 25: 311–316, 2004.
17. Giustarini D, Rossi R, Milzani A, Colombo R, and Dalle-Donne I. *S*-Glutathionylation: from redox regulation of protein functions to human diseases. *J Cell Mol Med* 8: 201–212, 2004.
18. Gu Z, Kaul M, Yan B, Kridel SJ, Cui J, Strongin A, Smith JW, Liddington RC, and Lipton SA. *S*-Nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. *Science* 297: 1186–1190, 2002.
19. Haendeler J, Hoffmann J, Tischler V, Berk BC, Zeiher AM, and Dimmeler S. Redox regulatory and anti-apoptotic functions of thioredoxin depend on *S*-nitrosylation at cysteine 69. *Nat Cell Biol* 4: 743–749, 2002.
20. Heo J and Campbell SL. Mechanism of $p21^{Ras}$ *S*-nitrosylation and kinetics of nitric oxide-mediated guanine nucleotide exchange. *Biochemistry* 43: 2314–2322, 2004.

21. Hess DT, Matsumoto A, Nudelman R, and Stamler JS. *S*-Nitrosylation: spectrum and specificity. *Nat Cell Biol* 3: E46–E49, 2001.
22. Hogg N. The kinetics of *S*-transnitrosation—a reversible second-order reaction. *Anal Biochem* 272: 257–262, 1999.
23. Hogg N. The biochemistry and physiology of *S*-nitrosothiols. *Annu Rev Pharmacol Toxicol* 42: 585–600, 2002.
24. Ji Y, Akerboom TPM, Sies H, and Thomas JA. *S*-Nitrosylation and *S*-glutathiolation of protein sulfhydryls by *S*-nitroso glutathione. *Arch Biochem Biophys* 362: 67–78, 1999.
25. Kim JE and Tannenbaum SR. *S*-Nitrosation regulates the activation of endogenous procaspase-9 in HT-29 human colon carcinoma cells. *J Biol Chem* 279: 9758–9764, 2004.
26. Klatt P and Lamas S. Regulation of protein function by *S*-glutathiolation in response to oxidative and nitrosative stress. *Eur J Biochem* 267: 4928–4944, 2000.
27. Konorev EA, Kalyanaraman B, and Hogg N. Modification of creatine kinase by *S*-nitrosothiols: *S*-nitrosation vs. *S*-thiolation. *Free Radic Biol Med* 28: 1671–1678, 2000.
28. Kuncewicz T, Sheta EA, Goldknopf IL, and Kone BC. Proteomic analysis of *S*-nitrosylated proteins in mesangial cells. *Mol Cell Proteomics* 2: 156–163, 2003.
29. Liu L, Hausladen A, Zeng M, Que L, Heitman J, and Stamler JS. A metabolic enzyme for *S*-nitrosothiol conserved from bacteria to humans. *Nature* 410: 490–494, 2001.
30. Mannick JB and Schonhoff CM. NO means no and yes: regulation of cell signaling by protein nitrosylation. *Free Radic Res* 38: 1–7, 2004.
31. Mannick JB, Schonhoff C, Papeta N, Ghafourifar P, Szibor M, Fang K, and Gaston B. *S*-Nitrosylation of mitochondrial caspases. *J Cell Biol* 154: 1111–1116, 2001.
32. Martinez-Ruiz A and Lamas S. Detection and proteomic identification of *S*-nitrosylated proteins in endothelial cells. *Arch Biochem Biophys* 423: 192–199, 2004.
33. Martinez-Ruiz A and Lamas S. *S*-nitrosylation: a potential new paradigm in signal transduction. *Cardiovasc Res* 62: 43–52, 2004.
34. Mohr S, Hallak H, Boitte A, Lapetina EG, and Brune B. Nitric oxide-induced *S*-glutathionylation and inactivation of glyceraldehyde-3-phosphate dehydrogenase. *J Biol Chem* 274: 9427–9430, 1999.
35. Nulton-Persson AC, Starke DW, Mieyal JJ, and Szweda LI. Reversible inactivation of alpha-ketoglutarate dehydrogenase in response to alterations in the mitochondrial glutathione status. *Biochemistry* 42: 4235–4242, 2003.
36. Padgett CM and Whorton AR. Cellular responses to nitric oxide: role of protein *S*-thiolation/dethiolation. *Arch Biochem Biophys* 358: 232–242, 1998.
37. Park HS, Huh SH, Kim MS, Lee SH, and Choi EJ. Nitric oxide negatively regulates c-Jun N-terminal kinase/stress-activated protein kinase by means of *S*-nitrosylation. *Proc Natl Acad Sci U S A* 97: 14382–14387, 2000.
38. Patel RP, Hogg N, Spencer NY, Kalyanaraman B, Matalon S, and Darley-Usmar VM. Biochemical characterization of human *S*-nitrosohemoglobin. Effects on oxygen binding and transnitrosation. *J Biol Chem* 274: 15487–15492, 1999.
39. Rassaf T, Bryan NS, Kelm M, and Feelisch M. Concomitant presence of *N*-nitroso and *S*-nitroso proteins in human plasma. *Free Radic Biol Med* 33: 1590–1596, 2002.
40. Rassaf T, Feelisch M, and Kelm M. Circulating NO pool: assessment of nitrite and nitroso species in blood and tissues. *Free Radic Biol Med* 36: 413–422, 2004.
41. Ravi K, Brennan LA, Levic S, Ross PA, and Black SM. *S*-Nitrosylation of endothelial nitric oxide synthase is associated with monomerization and decreased enzyme activity. *Proc Natl Acad Sci U S A* 101: 2619–2624, 2004.
42. Reynaert NL, Ckless K, Korn SH, Vos N, Guala AS, Wouters EF, van der Vliet A, and Janssen-Heininger YM. Nitric oxide represses inhibitory kappaB kinase through *S*-nitrosylation. *Proc Natl Acad Sci U S A* 101: 8945–8950, 2004.
43. Rizzo MA and Piston DW. Regulation of beta cell glucokinase by *S*-nitrosylation and association with nitric oxide synthase. *J Cell Biol* 161: 243–248, 2003.
44. Saville B. A scheme for the colorimetric determination of microgram amounts of thiols. *Analyst* 83: 670–672, 1958.
45. Stadtman ER and Levine RL. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids* 25: 207–218, 2003.
46. Stamler JS, Toone EJ, Lipton SA, and Sucher NJ. (*S*)NO signals: translocation, regulation, and a consensus motif. *Neuron* 18: 691–696, 1997.
47. Stamler JS, Lamas S, and Fang FC. Nitrosylation. the prototypic redox-based signaling mechanism. *Cell* 106: 675–683, 2001.
48. Tao L and English AM. Protein *S*-glutathiolation triggered by decomposed *S*-nitrosoglutathione. *Biochemistry* 43: 4028–4038, 2004.
49. Taylor ER, Hurrell F, Shannon R, Lin TK, Hirst J, and Murphy MP. Reversibile glutathionylation of complex I increases mitochondrial superoxide formation. *J Biol Chem* 278: 19603–19610, 2003.
50. Xian M, Chen X, Liu Z, Wang K, and Wang PG. Inhibition of papain by *S*-nitrosothiols. *J Biol Chem* 275: 20467–20473, 2000.
51. Zhang Y and Hogg N. Formation and stability of *S*-nitrosothiols in RAW 264.7 cells. *Am J Physiol Lung Cell Mol Physiol* 287: L467–L474, 2004.
52. Zhang Y and Hogg N. The mechanism of transmembrane *S*-nitrosothiol transport. *Proc Natl Acad Sci U S A* 101: 7891–7896, 2004.

Address reprint requests to:
Isabella Dalle-Donne, Ph.D.
Department of Biology
University of Milan
Via Celoria 26
I-20133 Milan, Italy

E-mail: quack@unimi.it

Received for publication October 22, 2004; accepted January 19, 2005.

This article has been cited by:

1. Wagner L. Batista , Fernando T. Ogata , Marli F. Curcio , Rodrigo B. Miguel , Roberto J. Arai , Alisson L. Matsuo , Miriam S. Moraes , Arnold Stern , Hugo P. Monteiro . S-Nitrosoglutathione and Endothelial Nitric Oxide Synthase-Derived Nitric Oxide Regulate Compartmentalized Ras S-Nitrosylation and Stimulate Cell Proliferation. *Antioxidants & Redox Signaling*, ahead of print. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links] [Supplemental material]
2. Wenwu Liu, Dong Wang, Kan Liu, Xuejun Sun. 2012. Nrf2 as a converging node for cellular signaling pathways of gasotransmitters. *Medical Hypotheses* **79**:3, 308-310. [CrossRef]
3. Mirko Zaffagnini , Mariette Bedhomme , Christophe H. Marchand , Samuel Morisse , Paolo Trost , Stéphane D. Lemaire . 2012. Redox Regulation in Photosynthetic Organisms: Focus on Glutathionylation. *Antioxidants & Redox Signaling* **16**:6, 567-586. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
4. Mirko Zaffagnini, Mariette Bedhomme, Stéphane D. Lemaire, Paolo Trost. 2012. The emerging roles of protein glutathionylation in chloroplasts. *Plant Science* . [CrossRef]
5. Karina van der Linde, Nora Gutsche, Hans-Martin Leffers, Christian Lindermayr, Bernd Müller, Simone Holtgrefe, Renate Scheibe. 2011. Regulation of plant cytosolic aldolase functions by redox-modifications. *Plant Physiology and Biochemistry* **49**:9, 946-957. [CrossRef]
6. Ying Xiong , Joachim D. Uys , Kenneth D. Tew , Danyelle M. Townsend . 2011. S-Glutathionylation: From Molecular Mechanisms to Health Outcomes. *Antioxidants & Redox Signaling* **15**:1, 233-270. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
7. Arthur JL Cooper, John T Pinto, Patrick S Callery. 2011. Reversible and irreversible protein glutathionylation: biological and clinical aspects. *Expert Opinion on Drug Metabolism & Toxicology* **7**:7, 891-910. [CrossRef]
8. Daniela Giustarini, Dimitrios Tsikas, Ranieri Rossi. 2011. Study of the effect of thiols on the vasodilatory potency of S-nitrosothiols by using a modified aortic ring assay. *Toxicology and Applied Pharmacology* . [CrossRef]
9. Claudia A. Staab, Tereza Hartmanová, Yasser El-Hawari, Bettina Ebert, Michael Kisiela, Vladimir Wsol, Hans-Jörg Martin, Edmund Maser. 2011. Studies on reduction of S-nitrosoglutathione by human carbonyl reductases 1 and 3. *Chemico-Biological Interactions* **191**:1-3, 95-103. [CrossRef]
10. Rebecca S. Zee , Chris B. Yoo , David R. Pimentel , David H. Perlman , Joseph R. Burgoyne , Xiuyun Hou , Mark E. McComb , Catherine E. Costello , Richard A. Cohen , Markus M. Bachschmid . 2010. Redox Regulation of Sirtuin-1 by S-Glutathiolation. *Antioxidants & Redox Signaling* **13**:7, 1023-1032. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links] [Supplemental material]
11. Marli F. Curcio , Wagner L. Batista , Edlaine Linares , Fabio D. Nascimento , Miriam S. Moraes , , Roberta E. Borges , Jan Sap , Arnold Stern , Hugo P. Monteiro . 2010. Regulatory Effects of Nitric Oxide on Src Kinase, FAK, p130Cas, and Receptor Protein Tyrosine Phosphatase Alpha (PTP-#): A Role for the Cellular Redox Environment. *Antioxidants & Redox Signaling* **13**:2, 109-125. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links] [Supplemental material]
12. Molly M. Gallogly , David W. Starke , John J. Mieyal . 2009. Mechanistic and Kinetic Details of Catalysis of Thiol-Disulfide Exchange by Glutaredoxins and Potential Mechanisms of Regulation. *Antioxidants & Redox Signaling* **11**:5, 1059-1081. [Abstract] [Full Text PDF] [Full Text PDF with Links]
13. Isabella Dalle-Donne, Ranieri Rossi, Graziano Colombo, Daniela Giustarini, Aldo Milzani. 2009. Protein S-glutathionylation: a regulatory device from bacteria to humans. *Trends in Biochemical Sciences* **34**:2, 85-96. [CrossRef]
14. C LEHNER, H KERSCHBAUM, U LUTZMEINDL. 2009. Nitric oxide suppresses growth and development in the unicellular green alga Micrasterias denticulata. *Journal of Plant Physiology* **166**:2, 117-127. [CrossRef]
15. Xing-Huang Gao, Mariette Bedhomme, Laure Michelet, Mirko Zaffagnini, Stéphane D. LemaireChapter 12 Glutathionylation in Photosynthetic Organisms **52**, 363-403. [CrossRef]
16. John J. Mieyal , Molly M. Gallogly , Suparna Qanungo , Elizabeth A. Sabens , Melissa D. Shelton . 2008. Molecular Mechanisms and Clinical Implications of Reversible Protein S-Glutathionylation. *Antioxidants & Redox Signaling* **10**:11, 1941-1988. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
17. Daniela Giustarini, Isabella Dalle-Donne, Roberto Colombo, Aldo Milzani, Ranieri Rossi. 2008. Is ascorbate able to reduce disulfide bridges? A cautionary note. *Nitric Oxide* **19**:3, 252-258. [CrossRef]
18. Lakshmi Santhanam, Marjan Gucek, Tashalee R. Brown, Malini Mansharamani, Sungwoo Ryoo, Christopher A. Lemmon, Lewis Romer, Artin A. Shoukas, Dan E. Berkowitz, Robert N. Cole. 2008. Selective fluorescent labeling of S-nitrosothiols (S-FLOS): A novel method for studying S-nitrosation. *Nitric Oxide* **19**:3, 295-302. [CrossRef]

19. Immacolata Castellano, Maria Rosaria Ruocco, Francesca Cecere, Antimo Di Maro, Angela Chambery, Andzelika Michniewicz, Giuseppe Parlato, Mariorosario Masullo, Emmanuele De Vendittis. 2008. Glutathionylation of the iron superoxide dismutase from the psychrophilic eubacterium Pseudoalteromonas haloplanktis. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* **1784**:5, 816-826. [CrossRef]
20. Isabella Dalle–Donne , Aldo Milzani , Nicoletta Gagliano , Roberto Colombo , Daniela Giustarini , Ranieri Rossi . 2008. Molecular Mechanisms and Potential Clinical Significance of S-Glutathionylation. *Antioxidants & Redox Signaling* **10**:3, 445-474. [Abstract] [Full Text PDF] [Full Text PDF with Links]
21. Barbara J. Buckley, Sheng Li, A. Richard Whorton. 2008. Keap1 modification and nuclear accumulation in response to S-nitrosocysteine. *Free Radical Biology and Medicine* **44**:4, 692-698. [CrossRef]
22. Jorge M. Romero, Oscar A. Bizzozero. 2008. Intracellular glutathione mediates the denitrosylation of protein nitrosothiols in the rat spinal cord. *Journal of Neuroscience Research* NA-NA. [CrossRef]
23. Isabella Dalle-Donne, Ranieri Rossi, Daniela Giustarini, Roberto Colombo, Aldo Milzani. 2007. S-glutathionylation in protein redox regulation. *Free Radical Biology and Medicine* **43**:6, 883-898. [CrossRef]
24. Isabella Dalle-Donne, Marina Carini, Giulio Vistoli, Luca Gamberoni, Daniela Giustarini, Roberto Colombo, Roberto Maffei Facino, Ranieri Rossi, Aldo Milzani, Giancarlo Aldini. 2007. Actin Cys374 as a nucleophilic target of #,#-unsaturated aldehydes. *Free Radical Biology and Medicine* **42**:5, 583-598. [CrossRef]
25. 2007. Irreversible Inactivation of Glutaredoxins 1 and 2 by Peroxynitrite. *Bulletin of the Korean Chemical Society* **28**:1, 125-128. [CrossRef]
26. Ernst van Faassen, Anatoly F. VaninLow-molecular-weight S-nitrosothiols 173-199. [CrossRef]
27. Pamela Maher . 2006. Redox Control of Neural Function: Background, Mechanisms, and Significance. *Antioxidants & Redox Signaling* **8**:11-12, 1941-1970. [Abstract] [Full Text PDF] [Full Text PDF with Links]
28. Claus Jacob, Iona Knight, Paul G. Winyard. 2006. Aspects of the biological redox chemistry of cysteine: from simple redox responses to sophisticated signalling pathways. *Biological Chemistry* **387**:10_11, 1385-1397. [CrossRef]
29. Q SHI, H CHEN, Y LOU. 2006. Further evidence that rat liver microsomal glutathione transferase 1 is not a cellular protein target for S-nitrosylation. *Chemico-Biological Interactions* **162**:3, 228-236. [CrossRef]
30. M KHAN, M JATANA, C ELANGO, A SINGHPAINTLIA, A SINGH, I SINGH. 2006. Cerebrovascular protection by various nitric oxide donors in rats after experimental stroke. *Nitric Oxide* **15**:2, 114-124. [CrossRef]
31. Jorge M. Romero, Oscar A. Bizzozero. 2006. Extracellular S-nitrosoglutathione, but not S-nitrosocysteine or NO, mediates protein S-nitrosation in rat spinal cord slices. *Journal of Neurochemistry* **99**:4, 1299. [CrossRef]
32. Philip Eaton , Michael J. Shattock . 2005. Protein S-Thiolation: Emphasis on Cell Signaling and Gene Expression. *Antioxidants & Redox Signaling* **7**:7-8, 839-840. [Citation] [Full Text PDF] [Full Text PDF with Links]