

S-Nitrosylation and S-Glutathiolation of Protein Sulfhydryls by S-Nitroso Glutathione¹

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Received July 20, 1998, and in revised form October 29, 1998

The modification of reactive protein sulfhydryls by S-nitrosoglutathione and other NO donors has been studied by gel isoelectric focusing. S-nitrosylated, unmodified, and S-glutathiolated protein forms are differentiated by this method. With specific antibodies for the protein of interest, both S-nitrosylation and S-glutathiolation of the protein were analyzed in mixtures obtained as soluble tissue or cell extracts. The effect of S-nitrosoglutathione (GSNO) on purified phosphorylase b, on carbonic anhydrase III in an extract from rat liver, and on H-ras expressed in *Escherichia coli* was examined. When fresh GSNO reacted with pure phosphorylase b, only S-nitrosylated forms of the protein were observed. Likewise the NO donors, amyl nitrite, spermine NONOate, and diethylamine NONOate, all generated S-nitrosylated phosphorylase b. When crude mixtures of proteins from rat liver (containing carbonic anhydrase III) or from *E. coli* (containing an overexpressed form of H-ras) were exposed to fresh GSNO, both the S-nitrosylated and the S-glutathiolated forms of the proteins were observed. It is suggested that reactive intermediates from the breakdown of GSNO are responsible for the observed S-glutathiolation. These experiments show that both S-nitrosylated and S-glutathiolated forms of proteins may be generated by the addition of GSNO to mixtures containing proteins with reactive sulfhydryls. These protein modifications may exhibit metabolic consequences independent of the release of nitric oxide.

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Proteins such as hemoglobin (1), albumin (2), glyceraldehyde-3-phosphate dehydrogenase (3), caspases (4), ras (5), alcohol dehydrogenase (6), and the Janus kinases (7) may be oxidatively modified to the S-nitrosylated form *in vivo*. In each case, the suggestion that the protein is regulated *in vivo* by formation of the S–NO protein adduct is primarily based on experiments showing that S-nitrosylation of the protein *in vitro* alters its activity. The agents that caused S-nitrosylation of protein sulfhydryls also were able to alter the activity of the proteins in intact cells. This evidence provides a very suggestive case that S-nitrosylation causes the observed cellular activity change *in vivo*. Reagents used to generate NO in experiments with intact cells are oxidative in nature and, in fact, the addition of NO to a protein sulfhydryl constitutes a one-electron oxidation of the sulfhydryl (8, 9). A direct evaluation of protein S-nitrosylation in intact cells is needed because oxidative modification of protein sulfhydryls by agents that produce S-nitrosylation *in vitro* may also result in either S-thiolation (10, 11) or even irreversible oxidative damage to protein sulfhydryls (12, 13).

S-Nitrosoglutathione (GSNO)³ is one of the important forms of nitric oxide *in vivo* (14–16), and it has frequently been used to study nitric oxide effects in intact cells (17–19). It has been suggested that GSNO is an effective agent for transfer of nitric oxide to other thiols including proteins by transnitrosylation. However, other potential protein modification reactions generated by this agent *in vivo* are not well studied.

Oxidative modification of protein sulfhydryls by S-glutathiolation occurs both *in vitro* and *in vivo* for proteins such as carbonic anhydrase III (20), creatine

¹ Journal Paper No. J-18025 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa.

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³ Abbreviations used: GSNO, S-nitrosoglutathione; NEM, N-ethylmaleimide; DTT, dithiothreitol; IAA, iodoacetic acid; IAM, iodoacetamide; PVDF, polyvinylidene fluoride.

kinase (21, 22), glyceraldehyde-3-phosphate dehydrogenase (23–25), actin (26), and glycogen phosphorylase b (22, 27). Since oxidative modification of protein sulfhydryls in intact cells occurs in the presence of a large pool of glutathione, many oxidative agents, including those derived from NO, have the potential to cause protein S-glutathiolation (28). Thus, at least some of the materials used to initiate NO modification reactions in cells may result in either S-nitrosylated or S-glutathiolated proteins *in vivo*.

The present report uses chemical modification procedures that can be combined with a gel isoelectric focusing method to assess oxidative modifications of protein sulfhydryls. Glycogen phosphorylase b is used as a model protein for these studies because it is known to have two reactive sulfhydryls per subunit that cause the homodimer to display up to five molecular forms, each differing by a single charge, during S-glutathiolation reactions (22, 27). If this protein is S-nitrosylated it would be expected to produce four different S-nitrosylated forms that do not differ in charge and are not separable on isoelectric focusing gels. However, these forms are separable when the protein is alkylated with iodoacetic acid, a negatively charged sulfhydryl-specific reagent. Using this method it is found that freshly prepared GSNO produces only S-nitrosylation of purified phosphorylase b, while partially decomposed GSNO contains materials that also generate S-glutathiolated forms of the protein. When freshly prepared GSNO was used to treat the mixture of proteins obtained as a soluble extract from *Escherichia coli* cells expressing H-ras, both S-glutathiolation and S-nitrosylation H-ras were observed. Similarly, when the soluble proteins from rat liver were treated with GSNO, both S-glutathiolated and S-nitrosylated forms of carbonic anhydrase III were observed. These results suggest that both the S-nitrosylated and S-glutathiolated forms of proteins may be generated when GSNO is used as a stimulatory agent.

MATERIALS AND METHODS

Materials

Glutathione, glutathione disulfide, dithiothreitol, iodoacetic acid, iodoacetamide, *N*-ethylmaleimide (NEM), glycogen phosphorylase b (from rabbit muscle), glutathione reductase (type III, from baker's yeast), NADPH, amyl nitrite, *N*-(1-naphthyl)ethylenediamine, and sulfanilamide were purchased from Sigma Chemical Company (St. Louis, MO); Spermine NONOate and diethylamine NONOate were from Cal-Biochem (La Jolla, CA); and ampholytes (pH 5–8 and pH 4–6) and GelBond PAG film were from Pharmacia LKB (Piscataway, NJ).

Reduced Glycogen Phosphorylase b

Glycogen phosphorylase b was reduced with 10 mM dithiothreitol in 20 mM β -glycerol phosphate (pH 7.0), 2 mM EDTA at 30°C for 30 min and dialyzed extensively against the same buffer for 24 h at 4°C. The protein was stored at –20°C in the same buffer containing 20%

glycerol as described (22, 27) at a concentration between 6 and 10 mg/ml. For modification reactions, the protein was diluted to 0.6 mg/ml or approximately 12 μ M reactive protein sulfhydryls (based on the presence of two reactive cysteines per protein subunit).

S-NitrosoGlutathione

GSNO was prepared by mixing 100 mM GSH and 100 mM sodium nitrite in 200 mM HCl at room temperature (29). The pH was adjusted to 7.40 with NaOH, and the solution was stored for short periods at 4°C. The concentration of GSNO was determined by absorbance at 334 nm, using the extinction coefficient 767 M⁻¹ cm⁻¹ (30).

Isoelectric Focusing Analysis of Protein S-Nitrosylation or S-Glutathiolation

S-Nitrosylated protein was analyzed by the Griess reagent method as described previously (31). All thiols in the samples were modified with 5 mM NEM before adding mercury.

Both the S-nitrosylated and S-thiolated forms of phosphorylase b were separated by thin-gel isoelectric focusing as described previously (22, 27). Protein samples in 20 mM β -glycerol phosphate (pH 7.0) were alkylated with 45 mM iodoacetamide to detect the S-glutathiolated forms of the protein. To detect the S-nitrosylated forms of the protein, a stock solution of iodoacetic acid was dissolved in 500 mM Tris base so that on adding to the protein sample the final concentration of iodoacetic acid was 45 mM and the sample pH was 7.8. (In order to get complete alkylation with iodoacetic acid it was necessary to use a higher pH.) Protein samples were treated for 15 min with the alkylating agent and 6 mg of protein was applied to each lane for separation on the isoelectric focusing gel (ampholyte pH range, 4–8). Quantitative S-nitrosylation or S-glutathiolation data were obtained by scanning the isoelectric focusing gel with a densitometer. The amount of S-nitrosylation or S-glutathiolation was calculated by dividing the density units for each protein band by the total density units of all protein bands in a single lane on the gel. Data are expressed as percentage of S-nitrosylation or S-glutathiolation.

The isoelectric focusing separation of H-ras and carbonic anhydrase III was analyzed by Western blotting as described previously (32, 33). Briefly, electrofocusing gels containing Netfix were prepared on GelBond PAG film as described. Gels were stripped from the Gelbond backing after focusing and trans-blotting to PVDF membranes by semidry transfer. Proteins were transferred in 0.35% acetic acid at constant voltage, 25 V for 1 h, nonspecific binding was blocked with 5% non-fat dry milk, and membranes were incubated with the appropriate primary antibody to detect the protein of interest. For H-ras, the monoclonal antibody to residues 157–181 (146-03E4; Quality Biotech, Camden, NJ) was diluted 1:3000. For carbonic anhydrase III a rabbit polyclonal antibody to the pure protein was prepared in our laboratory and then diluted 1:300. Each primary antibody was detected with either anti-mouse IgG conjugated with alkaline phosphatase (Sigma), or anti-rabbit IgG conjugated with alkaline phosphatase (Sigma), and the color was developed with 5-bromo-4-chloro-3-indolylphosphate and *p*-nitroblue tetrazolium chloride.

Determination of Glutathione and Related Compounds

The glutathione reductase method for determination of glutathione disulfide as described by Akerboom and Sies (34) was used to analyze preparations of GSNO.

GSNO, GSH, GSSG, and GSO₃H were determined by HPLC by modifications of the method described by Fariss and Reed (35). Samples were modified before separation on a 3-aminopropyl Spherisorb column as follows. Samples to be reduced were treated with 20

mM dithiothreitol at 30°C for 30 min. Subsequently both reduced and untreated samples were brought to 5% perchloric acid, the samples were divided in two and either 120 mM iodoacetamide or 120 mM iodoacetic acid was added to each. Excess KHCO_3 was added to make the pH basic, allowing the iodoacetamide or iodoacetic acid to alkylate free sulfhydryls. Samples were stored in the dark for 20 min and 1.5% FDNB (in EtOH) was added. The samples were stored at 4°C before separation on the HPLC.

Preparation of Protein Extracts Containing H-ras or Carbonic Anhydrase III

Preparation of a soluble protein extract from E. coli overexpressing the H-ras gene. *E. coli* strain JM 105 containing the pAT expression vector for H-ras (36) was grown at 37°C in Luria broth medium. At an optical density of 1.2–1.5 (600 nm), protein expression was induced by the adding 1 mM isopropyl- β -D-thiogalactopyranoside for 3 h. Cells were harvested by centrifugation at 16,000g for 10 min. The cells were washed once with 20 mM Tris-HCl (pH 7.2), 100 mM NaCl, 5 mM MgCl_2 and 1 mM phenylmethylsulfonyl fluoride. The cells were resuspended to 0.2 g of cell paste/ml with 20 mM β -glycerolphosphate-HCl (pH 7.0) and broken by sonication for 30 s. The soluble protein fraction was obtained by the centrifugation in a Beckman Airfuge Ultracentrifuge at 160,000g for 30 min.

Preparation of a soluble protein extract from rat liver. Male Sprague-Dawley rats (200–250 g), fed with Altromin standard diet *ad libitum*, were anesthetized with secobarbital sodium (100 mg/kg body wt) to obtain fresh liver tissue. The tissue was homogenized in a Potter homogenizer filled with ice-cold 10 mM Tris-HCl buffer (pH 7.2), 0.25 M sucrose. The homogenate was centrifuged at 4500g for 10 min (twice) and finally in a Beckman Airfuge Ultracentrifuge at 160,000g for 30 min to obtain the soluble protein extract.

RESULTS

An Electrofocusing Method for Analyzing Protein S-Nitrosylation

Two oxidative modifications of protein sulfhydryls, either S-glutathiolation or oxidation to a cysteic acid, increase the negative charge on a protein cysteine residue. On the other hand, oxidative modifications that produce S-cysteylation or S-nitrosylation are charge-silent. A method for analyzing charge-generating modifications by isoelectric focusing gel electrophoresis has been in use for some time. It is based on the alkylation of protein samples with a neutral reagent, iodoacetamide, to protect against artifactual sulfhydryl modification (10, 33). This method is illustrated in Fig. 1, where the S-glutathiolated forms of phosphorylase *b* are generated by reaction with glutathione disulfide (Fig. 1A). Because phosphorylase *b* is a homodimer with two reactive sulfhydryls per subunit, S-glutathiolation produces up to four acidic bands containing different amounts of disulfide-bonded glutathione (lane 2). The acidic protein forms are readily reduced by dithiothreitol, providing evidence for the covalent attachment of glutathione by a disulfide bond. Alkylation of these same protein samples with a negatively charged reagent, iodoacetate (lanes 3 and 4), prevents separation of the various protein species in the mixture

because all reactive cysteines have a similar negative charge.

Previously it was shown that S-cysteylated protein forms (disulfide-bound cysteine), a charge-silent modification, could be separated from unchanged protein by alkylating unmodified sulfhydryls with iodoacetate (27). All free sulfhydryls of phosphorylase *b* were converted to negatively charged species with this reagent, while sulfhydryls containing a disulfide-bound cysteine remained uncharged because they did not react with iodoacetate. Thus, S-cysteylation produced protein bands that migrated at a more alkaline *pI* when compared to unmodified protein bands. The experiments reported here are based on the premise that S-nitrosylated phosphorylase *b* (also a charge-silent modification) can be analyzed by similar methods. When phosphorylase *b* was modified by reaction with GSNO (Fig. 1B) this premise was verified by the observed bands. Lanes 1 and 2 show that unmodified and GSNO-treated phosphorylase *b* were not separated after alkylating the protein with iodoacetamide (lanes 1 and 2). However, when samples were alkylated with iodoacetate (lanes 3 and 4), there was a clear difference between the unmodified and GSNO-treated samples. The resulting bands in lane 4 were tentatively attributed to the S-nitrosylated forms of phosphorylase *b*. Part C of the figure again summarizes the effects of these sulfhydryl modifications. Thus, when samples are alkylated with iodoacetate the S-nitrosylated form of phosphorylase *b* would have no charge, while both S-glutathiolated and unmodified sulfhydryls would have a negative charge.

To obtain further evidence that the alkaline bands in lane 4 (Fig. 1B), resulted from S-nitrosylation of phosphorylase *b*, other NO-generating materials were used to modify the protein. We reasoned that alkaline bands with identical *pI*s should be identifiable after treatment with all reagents that generate NO. Figure 2 shows the results of modification reactions using equal concentrations of amyl nitrite, spermine NONOate, and diethylamine NONOate. In this experiment, samples were alkylated with iodoacetate (the exception is the left-most lane), and in addition each sample was reduced with dithiothreitol after reaction. All of the NO-donating materials produced isoforms with alkaline *pI*s. Because we did not attempt to optimize reaction conditions or to standardize the rates of NO production by these reagents, it is not possible to conclude which reagent is most effective. However, the *pI*s of the resulting protein bands were identical with each reagent, suggesting that the protein bands generated by these NO-donating agents resulted from S-nitrosylated forms of phosphorylase *b*. In each case the modification was sensitive to reduction with dithiothreitol (+DTT in the figure). Further evidence for S-nitrosy-

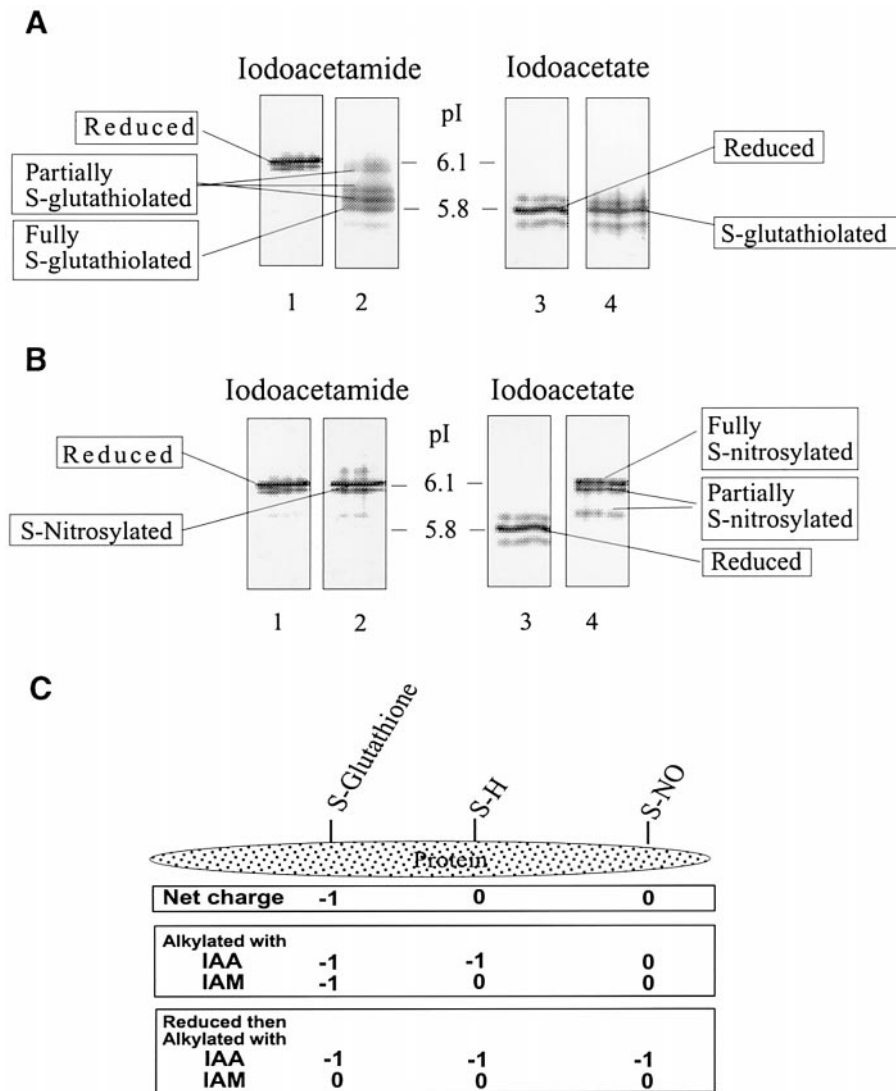


FIG. 1. Separation of S-nitrosylated and S-glutathiolated forms of glycogen phosphorylase *b* on isoelectric focusing gels. Modified forms of phosphorylase *b* were made by incubating fully reduced glycogen phosphorylase *b* (0.6 mg/ml, approx 12 μ M reactive protein sulfhydryls) with (A) 5.0 mM glutathione disulfide (GSSG) or (B) 1.0 mM freshly prepared S-nitrosoglutathione (GSNO) in 20 mM β -glycerophosphate (pH 7.0) at 37°C for 10 min. Reactions were stopped with 45 mM iodoacetamide (IAM) or iodoacetic acid (IAA) and separated by thin-gel electrophoresis as described under Materials and Methods. (C) Model for electrofocusing gels.

lation of phosphorylase *b* was obtained by quantitatively comparing the density of the modified protein bands on electrofocusing gels to the amount of S-nitrosylation as determined by the Griess reagent (Table I). A total of four protein samples, modified either by GSNO or spermine NONOate were compared. The electrofocusing data compared well with that obtained by the Griess reagent, substantiating the suitability of gel electrofocusing as a method for quantifying the amount of phosphorylase *b* S-nitrosylation.

Stability of S-Nitrosylated Phosphorylase *b*

In an effort to assess quantitative aspects of the electrofocusing method, it was important to establish the stability of the S-NO bond in S-nitrosylated phosphorylase *b*. Consequently, the S-nitrosylated form of phosphorylase *b* was prepared and stored in pH 7.0 buffer at either 4 or 30°C. Samples were analyzed by alkylation with IAA and separation on electrofocusing gels. After 24 h at 4°C, approximately 80% of the phosphorylase was unchanged, while at 30°C approximately 50% remained. After 2 h at 30°C there was little

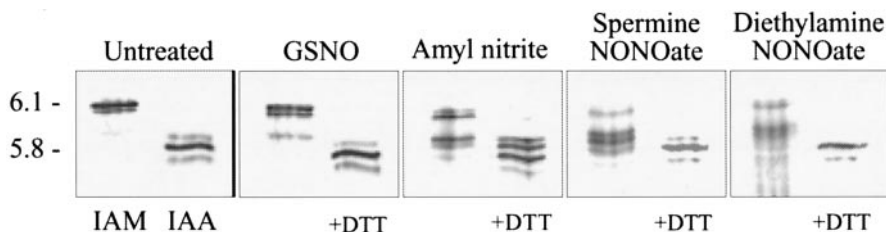


FIG. 2. S-nitrosylation of phosphorylase *b* by NO donors. Reduced protein (0.6 mg/ml, approx 12 μ M reactive protein sulfhydryls) was treated with 1 mM each of *S*-nitrosoglutathione (GSNO), amylnitrite, spermine NONOate, or diethylamine NONOate (DEA NONOate) in 20 mM β -glycerophosphate (pH 7.0) at 37°C for 10 min. After the incubation, one aliquot was stopped by adding 45 mM IAA, and a second was treated with 10 mM dithiothreitol (DTT) at 37°C for 20 min before alkylating with IAA. The left figure shows reduced protein, after alkylation with either 45 mM IAM or IAA.

evidence for decomposition. These data suggest that with proper care S-nitrosylated proteins may be analyzed by electrofocusing with little concern about decomposition.

A recent report on the crystal structure of GSNO-modified glutathione reductase showed that the protein contained the sulfenic form of an active site cysteine (37). Since protein sulfenic acids are easily reduced with dithiothreitol, while the sulfinic and sulfonic acids are not, we examined S-nitrosylated phosphorylase *b* for acidic protein bands that might

indicate the presence of one of these oxidized species. Phosphorylase *b* containing sulfenic acid modifications would have acidic bands after alkylation with IAM that were sensitive to reduction by dithiothreitol. Sulfinic and sulfonic acids would not be reduced. The S-nitrosylated protein used for our stability study had very little contamination with acidic bands at the start of the experiment. Samples incubated for 24 h at 4 or 30°C were alkylated and analyzed. In each case the sample contained substantial amounts of acidic forms that were entirely sensitive to dithiothreitol. These bands thus had the expected properties of a protein sulfenic acid as suggested by the crystal structure data of Becker *et al.* (37). Thus, S-nitrosylated phosphorylase *b* probably also contains some sulfenic acid derivatives after storage.

A second concern is the potential reaction of the S-nitrosylated protein with glutathione or other thiols. Any S-nitrosylated protein sample containing contaminating materials with reactive sulfhydryls could be inadvertently modified by transnitrosation during storage or processing. In addition, glutathione could potentially replace the NO on S-nitrosylated proteins by transnitrosation, thus converting the proteins to an S-glutathiolated protein form. Figure 3 examines these possibilities. Phosphorylase was derivatized with iodoacetamide to detect the potential formation of S-glutathiolated phosphorylase and with iodoacetate to detect S-nitrosylated forms. There was no evidence for the formation of S-glutathiolated phosphorylase *b* (Fig. 3, left). However, even molar equivalent concentrations of glutathione, i.e., 25 μ M glutathione with 12 μ M phosphorylase *b*, was sufficient to produce a 15–20% loss of S-nitrosylated phosphorylase in 10 min. At 100 μ M glutathione, the loss of S-nitrosylated phosphorylase was nearly complete. This experiment indicates that care must be taken to remove glutathione or other thiols from samples to be analyzed for S-nitrosylated protein. Iodoacetate is a convenient blocking agent for removal of this potential interference.

TABLE I

Comparison of Isoelectric Focusing Electrophoresis (IEF) and Spectrophotometric Analysis of S-Nitrosylated Phosphorylase *b*

	Calculated percentage of protein-S-NO			
	GSNO		Spermine NONOate	
	50 (μ M)	500 (μ M)	50 (μ M)	500 (μ M)
Isoelectric focusing	37 \pm 2	59 \pm 7	13 \pm 1	33 \pm 2
Spectrophotometry	43 \pm 7	69 \pm 6	18 \pm 1	32 \pm 2

Note. Phosphorylase *b* (0.6 mg/ml, approximately 12 μ M reactive protein sulfhydryls) was treated with either *S*-nitrosoglutathione (GSNO) or spermine NONOate at 37°C for 10 min in 20 mM β -glycerophosphate (pH 7.0) and the protein was dialyzed to remove low-molecular-weight reactants. IEF: Before the focusing, 45 mM iodoacetic acid was used to block those unmodified sulfhydryl groups. As shown in Fig. 1, S-nitrosylated protein was analyzed with a densitometer by scanning thin gel and the percentage of modified sulfhydryl groups was calculated as reduced protein was referenced as 100%. Spectrophotometry: Neutral Griess reagents were used to measure NO released from S-nitrosylated protein. As free thiol groups can interfere with this assay, the method was modified by treating samples with 5 mM *N*-ethylmaleimide to block the unmodified protein sulfhydryls before adding mercury chloride. The percentage of S-nitrosylated protein was shown as the concentration of protein S-nitrosothiols divided by that of total protein sulfhydryl group. Data are mean values \pm SE of three to seven experiments ($t > 0.05$).

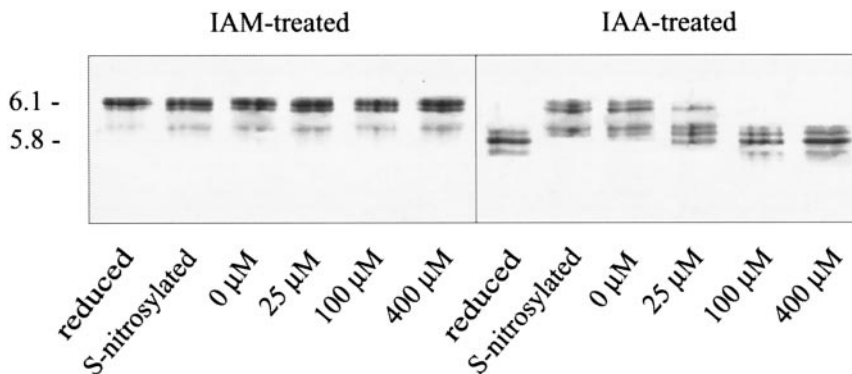


FIG. 3. Effect of GSH on stability of S-nitrosylated protein. S-nitrosylated phosphorylase *b* was prepared by treating fully reduced phosphorylase *b* (0.6 mg/ml, approx 12 μ M reactive protein sulfhydryls) with 50 μ M GSNO in 20 mM β -glycerophosphate (pH 7.0) at 37°C and reactants were removed by dialysis. The S-nitrosylated protein was incubated with GSH for 10 min at 37°C as indicated. The reaction was stopped by adding 45 mM IAM or IAA and the protein was analyzed by isoelectric focusing.

GSNO Reaction with Phosphorylase *b*

Figure 4 shows the time course and concentration dependence of the reaction of GSNO with phosphorylase *b*. This experiment demonstrates that S-nitrosylation was efficient, occurring at low concentrations of GSNO with just a slight molar excess of the reagent. When phosphorylase *b* (12 μ M in reactive protein sulfhydryls) was incubated with a 4-fold excess of GSNO (50 μ M) the reaction was rapid at pH 7.0 (Fig. 4A). The reaction was complete in approximately 20 min, when the protein was over 60% modified. The effect of GSNO concentration is examined in Fig. 4B, demonstrating significant amounts of S-nitrosylation with equimolar

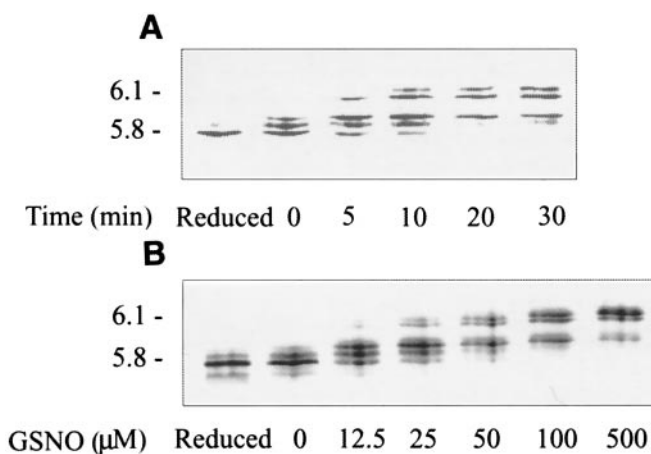


FIG. 4. Reaction of phosphorylase *b* with GSNO. (A) Fully reduced phosphorylase *b* (0.6 mg/ml, approx 12 μ M reactive protein sulfhydryls) was incubated with 50 μ M GSNO in 20 mM β -glycerophosphate (pH 7.0) at 37°C and aliquots were alkylated with 45 mM IAA at the times indicated to stop the reaction. Samples were analyzed by isoelectric focusing. (B) Reduced phosphorylase *b* was treated with varying concentrations of GSNO in 20 mM β -glycerophosphate (pH 7.0) at 37°C for 10 min.

GSNO (12.5 μ M) in just 10 min. Phosphorylase *b* was approximately 80% S-nitrosylated with 500 μ M (a 40-fold molar excess).

Since reactions between GSNO and protein sulfhydryls would normally occur in the presence of a large glutathione pool in cells, we examined the effect of glutathione on the reaction. Figure 5 shows that a twofold excess of glutathione (100 μ M) blocked this reaction effectively. Thus, GSNO shows little preference for the protein sites on phosphorylase *b* when compared to glutathione sulfhydryls. This experiment also gave no evidence for the intermediate formation of S-glutathiolated phosphorylase during the reaction (Fig. 5, left, samples alkylated with IAM). Since protein sulfhydryl concentrations in cells may be similar in concentration to the concentration of glutathione, this experiment suggests that protein S-nitrosylation and formation of S-nitrosoglutathione are both potentially important when NO is generated in intact cells. In addition, the direct formation of S-glutathiolated

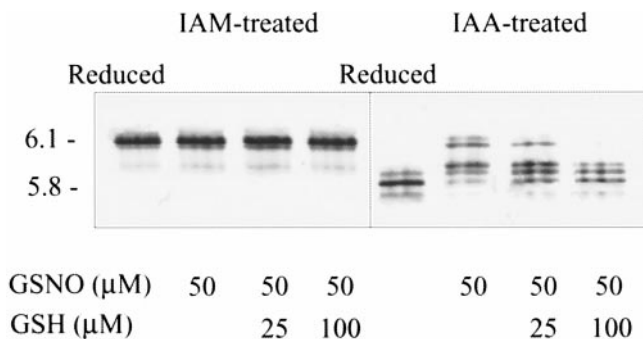


FIG. 5. Effect of glutathione on the reaction of phosphorylase *b* with GSNO. Reduced phosphorylase *b* (0.6 mg/ml, approx 12 μ M reactive protein sulfhydryls) was treated with 50 μ M GSNO in the presence of 25 or 100 μ M GSH in 20 mM β -glycerophosphate (pH 7.0) at 37°C for 10 min. IAM or IAA was added before focusing.

proteins as a consequence of NO-mediated protein oxidation does not seem likely.

GSNO may react with proteins by first decomposing to a reactive NO_x species or by a direct reaction and transfer of NO. If decomposition of GSNO to an NO_x form was prerequisite to the formation of S-nitrosylated phosphorylase *b*, decomposition of the GSNO solution, i.e., loss of GSNO, should occur at the same rate as the observed S-nitrosylation reaction. Thus, partially decomposed solutions of GSNO might be more effective at S-nitrosylating proteins. In order to explore the reactive forms of glutathione present in partially decomposed GSNO, an HPLC method was developed to identify modified forms of glutathione that might be present. Figure 6 shows a typical chromatogram from the GSNO solution after decomposition for 72 h at room temperature. The peaks are identified by identical retention times with standard substances. When samples were alkylated with iodoacetamide (Fig. 6A), derivatized sulfhydryl-containing molecules migrate more rapidly than when they are alkylated with iodoacetate (Fig. 6B) because the negative charge added by iodoacetate retards molecules on this column. In addition, when samples were treated with dithiothreitol before alkylation, susceptible bonds (disulfides and sulfenic acids) were converted to reduced sulfhydryls that can be derivatized by the reagents. Thus, a use of the two alkylating reagents combined with susceptibility to reduction by dithiothreitol permitted us to identify the following peaks by comparison to the retention times of pure materials: GSSG (14.2–14.4 min with either alkylating agent), GSH (7.8 min with iodoacetamide and 13.2 min with iodoacetic acid), and GSO₃H (13.3–13.4 min with either alkylating agent). Both GSH and GSO₃H had the same retention time when a reduced sample was alkylated with iodoacetate. GSNO (6.6–6.7 min with either alkylating agent) was identified by the fact that the peak was reduced to GSH with dithiothreitol. When alkylated with iodoacetamide, GSNO and GSH had different retention times, i.e., 6.6 min for GSNO and 7.8 min for GSH. Thus, it was possible to quantitate the amounts of GSNO, GSH, GSO₃H, and GSSG in samples of degraded GSNO. Since these materials did not account for all of the glutathione as the sample decomposed, other minor forms of glutathione were generated in the mixture. Figure 7 quantifies the amount of GSNO, GSSG, and GSO₃H obtained from the HPLC analysis of samples from the initial GSNO solution (0 time), the 72-h degraded sample, and the 120-h degraded samples. These three glutathione forms account for up to 95% of the total glutathione in the samples. In addition, Fig. 7 shows data on the GSNO and GSSG content of decomposed GSNO obtained by spectrophotometric analysis. The kinetics

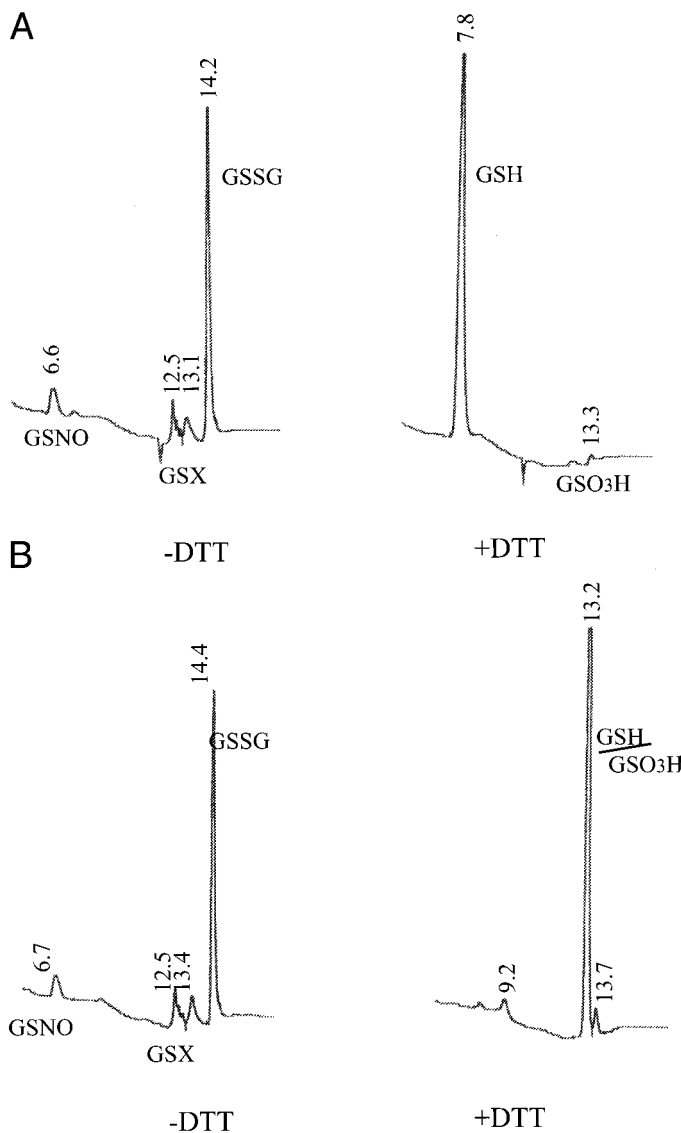


FIG. 6. HPLC analysis of glutathione derivatives. Freshly prepared 100 mM GSNO was kept at room temperature for 72 h and a sample was derivatized as follows for analysis on a 3-aminopropyl ion-exchange column as described under Materials and Methods. (A) treated with IAM. The sample was divided into two fractions and one was incubated for 20 min with 20 mM dithiothreitol (+DTT). Both samples were then alkylated with 120 mM iodoacetamide. The labels on the figure are assigned by identity to the retention times for the pure indicated substances. GSX indicates an unknown form of glutathione. (B) treated with IAA. A second sample was similarly divided into two fractions; one was reduced with DTT, and both samples were alkylated with 120 mM iodoacetic acid.

of this decomposition reaction are relatively slow in comparison to the rate at which protein S-nitrosylation occurs (Fig. 4). Since protein S-nitrosylation is much more rapid than the decomposition of GSNO to a reactive intermediate, it is apparent that S-nitrosylation occurs by transnitrosation.

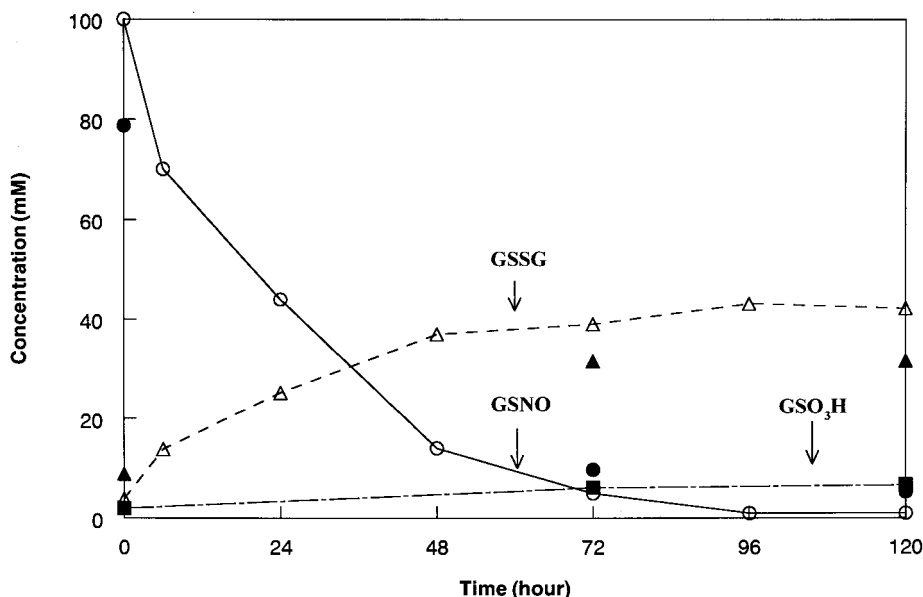


FIG. 7. Decomposition of GSNO with formation of GSSG and GSO₃H. Freshly prepared 100 mM GSNO was kept at room temperature for 120 h and samples were removed at the indicated times for analysis. The following analyses were made: (○) GSNO measured at 334 nm using an extinction coefficient of 767 M⁻¹cm⁻¹, (△) GSSG determined with GSH reductase as described under Materials and Methods. The following data points in the figure were determined by the HPLC method: (■) glutathione sulfonate, (▲) glutathione disulfide, and (●) GSNO.

When HPLC samples were alkylated with either iodoacetamide or iodoacetate, unidentified peaks were observed at 12.5 and 13.1 min without reduction (Fig. 6). Since these disappeared when samples were reduced (Fig. 6A), it appears that these negatively charged derivatives were readily reduced. One molecular species that might have these properties is the sulfenic acid of glutathione. On reduction it would be converted into GSH and be indistinguishable from that molecule. This form of glutathione might effectively react with protein sulfhydryls to form an S-glutathiolated protein.

In order to determine the effect of degraded GSNO on protein sulfhydryl modification, phosphorylase *b* was exposed to either newly prepared GSNO or a 72 h-degraded sample of the compound. Since both glutathione disulfide and potential reactive forms such as glutathione sulfenic acid might be present, Fig. 8A shows an experiment designed to detect the S-glutathiolated forms of phosphorylase *b*. Since the decomposed GSNO contained enough GSSG to contribute approximately 40 μ M GSSG to the reaction mixture (Fig. 7), control reactions with pure GSSG were used to assess the importance of the GSSG contamination in decomposed GSNO. With 0.1 mM GSSG there was no evidence of S-glutathiolation, but with 5 mM GSSG there was extensive S-glutathiolation. Thus, the 40 μ M GSSG contaminant in the decomposed GSNO was insufficient to contribute to the observed S-glutathiolation. It is proposed that S-glutathiolation resulted from reactive glutathione intermediates such as glutathione

sulfenic acid, i.e., to the material designated GSX in Fig. 6. Figure 8B shows the S-nitrosylated forms that resulted from reaction with fresh or decomposed GSNO. Decomposed GSNO was a much less effective S-nitrosylating reagent, since the decomposed GSNO contained less than 10% of the original GSNO. S-glutathiolated forms of phosphorylase *b* were not detected in this experiment as demonstrated by the lack of modification by GSSG.

Figure 9 shows the extent of protein S-glutathiolation and S-nitrosylation with samples of GSNO decomposed for the times indicated. The extent of each of these modifications correlates well with the concentration of GSX (S-glutathiolation) and GSNO (S-nitrosylation). Since GSNO decomposition may contribute to the generation of S-glutathiolated proteins, it seems possible that the effects of GSNO in intact cells may include both S-nitrosylation and S-glutathiolation of protein sulfhydryls.

Protein Modification Resulting from GSNO Treatment of Crude Protein Mixtures

The mixture of proteins in soluble extracts from cells and tissues is an appropriate model to examine the potential effects of GSNO on cellular proteins. A method is currently available to analyze this possibility (32), since Western blots of electrofocusing gels can provide data for individual proteins in the mixture like that already obtained with purified phosphorylase *b*. Both rat liver carbonic anhydrase III and H-ras can be

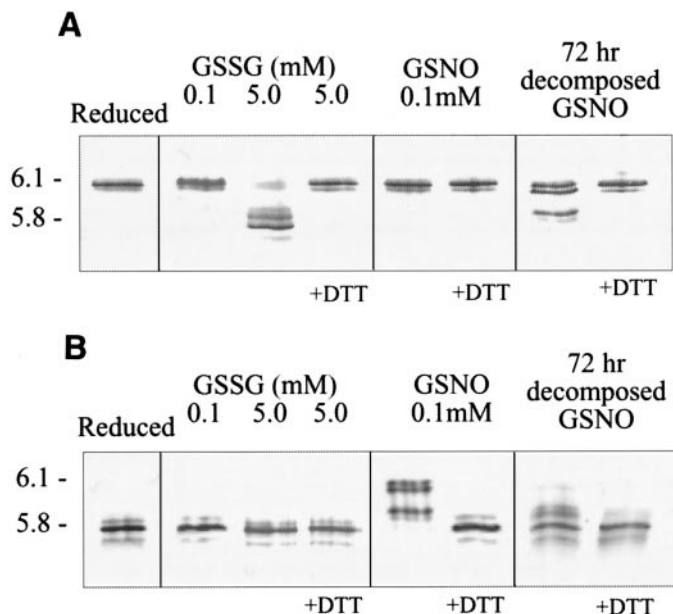


FIG. 8. Comparison of the modified forms of phosphorylase *b* generated by reaction with GSSG, fresh GSNO, or decomposed GSNO. Reduced phosphorylase *b* was separately treated with 0.1 and 5.0 mM GSSG, 100 μ M freshly synthesized GSNO, or 72-h-decomposed GSNO (see Fig. 7) in 20 mM β -glycerophosphate (pH 7.0) at 37°C for 10 min. (Equal volumes of 100 mM fresh GSNO and 72-h-decomposed GSNO were added to protein solutions to achieve the appropriate incubation conditions for this experiment. Therefore, the reaction with decomposed GSNO contained very little GSNO and approximately 40 μ M GSSG.) (A) IAM was used to stop the reaction in order to observe negatively charged forms of phosphorylase *b* (S-glutathiolated) that might be generated during reaction. (B) A second aliquot of the reaction mixture was alkylated with IAA to stop the reaction. Therefore S-nitrosylated forms of phosphorylase *b* were detected on isoelectric focusing gels. For each reaction, aliquots were treated with 10 mM DTT to demonstrate the reversible nature of the modification reaction.

analyzed by this method. Protein mixtures are separated by gel electrofocusing, the gel is transferred to PVDF membranes, and a specific antibody to the protein of interest is used to visualize the *pI* of all molecular forms of that specific protein. For the present experiment GSNO was used to modify both a soluble extract from rat liver containing normal amounts of carbonic anhydrase III and a soluble extract from *E. coli* that contained an expressed wild-type H-ras. Fresh GSNO was used for each experiment since the experiments with phosphorylase *b* suggested that only S-nitrosylation of each protein would occur if there were no decomposition of GSNO to other reactive materials.

Figure 10 shows data obtained with protein extracts from male rat liver in which approximately 5–10% of the protein is normally carbonic anhydrase III (38). This protein has two reactive sulfhydryls that can be S-glutathiolated to produce acidic forms of the protein. Western blots of liver tissue extracts treated with io-

doacetamide, using a polyclonal antibody to carbonic anhydrase III normally reveal three isoforms of the protein with *pI*s of 7.0 (fully reduced), 6.4 (one site S-glutathiolated), and 6.1 (both sites glutathiolated). The data on the left side of the figure (samples derivatized with IAM) reveal the S-glutathiolated forms of carbonic anhydrase in samples that were treated with diamide (an agent used frequently to stimulate protein mixed-disulfide formation) and also in samples that were treated with GSNO. Thus, fresh GSNO clearly generates significant amounts of the S-glutathiolated form of carbonic anhydrase III. The right side of this figure (samples alkylated with IAA) shows that GSNO also produced a significant amount of S-nitrosylation of this protein. IAA did not react with both sulfhydryls of the carbonic anhydrase III since only the *pI* 6.4 isoform of the protein was produced on addition of IAA. This fact clearly shows a difference in the reactivity of these two sulfhydryls. Taken together it appears that GSNO converted nearly all of the most reactive cysteine in carbonic anhydrase III into either the S-glutathiolated or the S-nitrosylated form.

Figure 11 shows a similar experiment with a protein extract from *E. coli* expressing H-ras. Since protein modification of the reactive cysteines of H-ras has not been previously studied in depth, the effects of S-glutathiolating agents and S-nitrosylating agents on the pure protein were examined (Fig. 11A). The reduced form of pure H-ras had a *pI* of 4.3 but since it was not completely pure a minor contaminating band with a *pI* of 4.1 was observed in the sample treated with iodoacetamide. When S-glutathiolated with diamide, the protein migrated as a mixture of up to four more acidic bands. These bands were sensitive to reduction by dithiothreitol with the reappearance of the original band at *pI* at 4.3 (data not shown here). The data are consistent with the presence of four reactive cysteines in the protein. This pure protein was produced by expression in *E. coli* where lipidation does not occur. Therefore, the reactive cysteines are cys 118 (normally free and reactive), cys 181 and 184 (normally extensively palmitoylated in mammalian cells), and cys 186 (normally prenylated in mammalian cells). When the pure protein was alkylated with IAM, the primary protein band occurred at pH 3.9, consistent with reaction at three of the potentially reactive cysteines. When treated with GSNO, the protein was clearly S-nitrosylated, producing bands with *pI*s of 4.3 (completely modified), 4.1, and 4.0 (partially S-nitrosylated). In experiments not shown evidence of S-glutathiolated H-ras was found on alkylating with IAM after the reaction with fresh GSNO.

Figure 11B shows the effect of GSNO on the H-ras in a soluble preparation of the proteins from *E. coli* in which the pAT vector was present, thereby expressing significant amounts of soluble, unlipidated H-ras. The

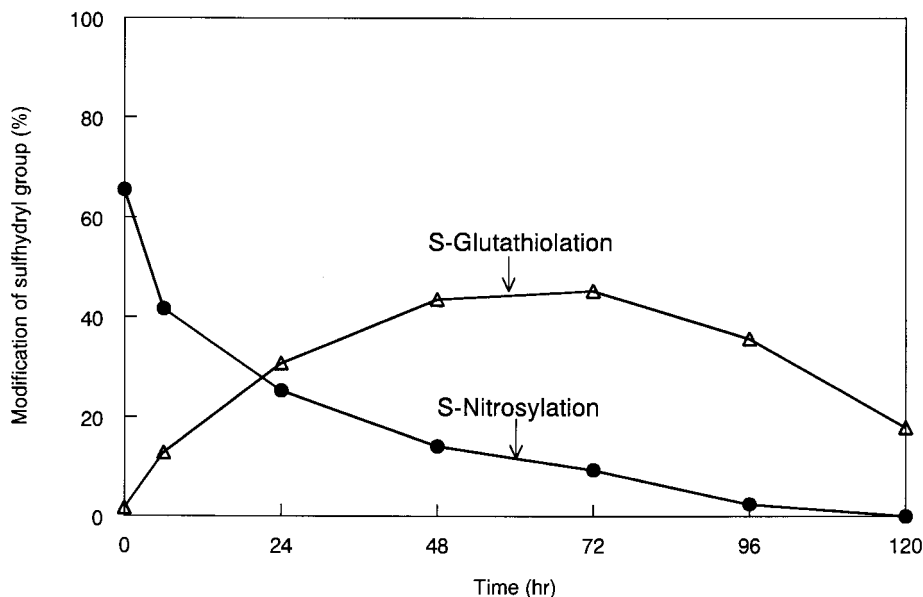


FIG. 9. Time-dependent decomposition of *S*-nitrosoglutathione produces increased *S*-thiolation and decreased *S*-nitrosylation of phosphorylase *b*. Newly synthesized GSNO was incubated at room temperature and, at the indicated time, an aliquot of the GSNO solution was added to reduced phosphorylase *b* (0.6 mg/ml, approximately 12 μ M reactive protein sulfhydryls) in 20 mM β -glycerophosphate (pH 7.0) at 37°C for 10 min. The reaction with phosphorylase *b* was stopped by adding either 45 mM IAM or 45 mM IAA before separating the modified protein bands by isoelectric focusing. The extent of either *S*-glutathiolation (samples treated with iodoacetamide) or *S*-nitrosylation (samples treated with iodoacetic acid) was assessed by densitometry. Data are reported as the percentage of maximum modification, assuming that there are four modification sites per molecule of phosphorylase *b* dimer.

samples alkylated with IAM give clear evidence of significant, dithiothreitol-sensitive *S*-glutathiolation of the H-ras, while samples alkylated with IAA also show significant amounts of dithiothreitol-sensitive *S*-nitrosylation. Thus, fresh GSNO produced both *S*-glutathiolation and *S*-nitrosylation of a protein with reactive cysteines in the soluble mixture of proteins from *E. coli* cells.

DISCUSSION

Interest in the reaction of NO and other NO-containing molecules such as GSNO, or peroxyxynitrite with protein sulfhydryls, derives from many reports of the biologically significant effects generated in intact cells by such molecules. No matter what NO donor is present outside cells, it seems certain that GSNO is a major participant in the intracellular milieu (14–16). Thus, the protein modification reactions generated by GSNO have potentially important biological consequences. One possible consequence of intracellular pools of GSNO is the transient generation of *S*-nitrosylated proteins by a simple transnitrosation reaction (39, 40). Indeed, the fact that pure proteins with reactive sulfhydryls are readily *S*-nitrosylated by such reactions provides strong support that this modification occurs in intact cells (1–7). This paper reports a methodological approach that may be useful for studying the

occurrence of protein *S*-nitrosylation in cells and the possible role this may play in the biological roles of nitric oxide. Figures 10 and 11 demonstrate that both *S*-nitrosylation and *S*-glutathiolation (oxidative modifications that both have physiological significance) can be revealed when specific proteins are analyzed by nondenaturing isoelectric focusing on gels that can then be visualized by antibody-based protocols specific for the protein of interest. In the illustrated experiments it was possible to show that a simple reaction with GSNO may produce more complex protein modifications than originally thought. It appears that GSNO may give rise to reactive materials that can both *S*-thiolate and *S*-nitrosylate reactive cysteines. This process may result from degradation of GSNO into either or both GSSG and the sulfenic acid of glutathione as illustrated by the reported experiments with pure glycogen phosphorylase *b* (see Figs. 7–9).

In order to understand the potential role of *S*-nitrosylated proteins in intact cells we explored the stability of *S*-nitrosylated phosphorylase *b* and the influence of glutathione on both the formation and stability of that modification. Since even minimal concentrations of glutathione can prevent reaction of GSNO with phosphorylase *b* (Fig. 5), it does not seem likely that a trans-nitrosylation reaction from GSNO to a reactive protein sulfhydryl has a high probability in intact cells.

Similarly, if S-nitrosylated proteins are generated in cells, it seems probably that reaction with the glutathione pool results only in the formation of GSNO and reduced protein sulfhydryls (Fig. 3). Thus, it does not appear that an S-glutathiolated intermediate protein form plays a role in the trans-nitrosylation reaction (Fig. 3). These results suggest that S-nitrosylated proteins may be quite transient in cells.

However, it is possible for GSNO to generate other reactive species that may play a significant role in cellular protein modification reactions (41, 42). Thus, it is known that transition metal ions and other molecules may catalyze the rapid generation of other reactive species from GSNO (42). In our experiments it appears that these materials may be responsible for the generation of S-glutathiolated proteins from reactive materials such as glutathione sulfenic acid or from less reactive materials such as GSSG (Figs. 8 and 9). Thus, we propose that intracellular GSNO may play a role in the formation of two different oxidative modifications of protein sulfhydryls, i.e., both S-glutathiolation and S-nitrosylation.

GSNO is only one of several S-nitrosothiols that have been used to manipulate the metabolism of intact cells. Our experiments suggest that each of these materials may have metabolic consequences that do not result from the simple generation of free NO. As an example, when cysteine-NO is used, the cysteine moiety of the molecule may have significant protein modulatory effects through the formation of high concentrations of

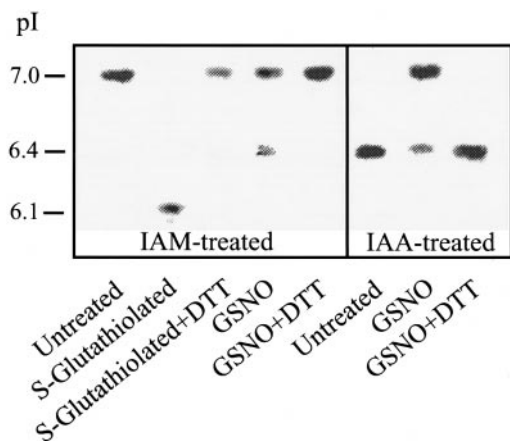


FIG. 10. Reaction of GSNO with carbonic anhydrase III. A soluble protein extract from rat liver was prepared in 20 mM β -glycerophosphate buffer (pH 7.0). The proteins were treated as follows: S-glutathiolated, 2 mM GSH and 4 mM diamide at room temperature for 20 min; S-glutathiolated + DTT, as described and then treated with 10 mM DTT at 37°C for 15 min; GSNO, 5 mM GSNO at 37°C for 10 min; GSNO + DTT, as described and then treated with 10 mM DTT at 37°C for 20 min. Samples were alkylated with 40 mM IAM or IAA as shown in the figure. After electrofocusing, proteins were transferred to a PVDF membrane, and carbonic anhydrase III was visualized with a polyclonal anti-CA III antibody as previously described (37).

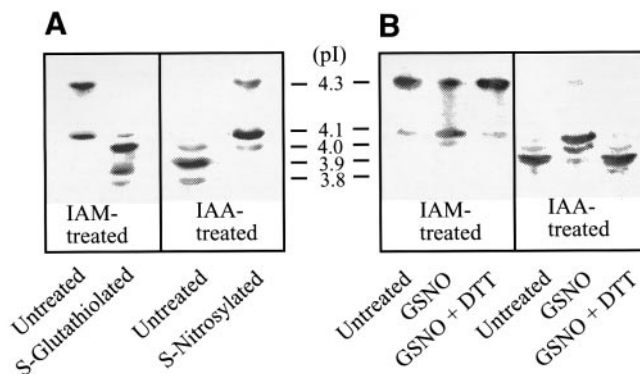


FIG. 11. Reaction of GSNO with H-Ras. (A) Purified H-ras in 20 mM β -glycerophosphate buffer (pH 7.0) was treated as follows: S-glutathiolated, 1 mM GSH and 2 mM diamide at room temperature for 15 min; S-nitrosylated, 1 mM GSNO at 37°C for 10 min. The samples were alkylated with 40 mM IAM or IAA as shown in the figure before the focusing and protein bands were visualized by Coomassie blue. (B) The soluble protein extract in 20 mM β -glycerophosphate buffer (pH 7.0) from *E. coli* cells expressing a pAT expression vector for H-ras (10) was treated directly as follows: GSNO, 2 mM GSNO at 37°C for 10 min; GSNO + DTT, after reaction with GSNO the extract was treated with 10 mM DTT at 37°C for 20 min. Samples were alkylated with either 40 mM IAM or IAA before focusing. Protein bands were transferred to a PVDF membrane and the H-ras in the cell extract was visualized with anti-H-Ras monoclonal antibody 146-3E4 (NCI Repository at Quality Biotech) as described under Materials and Methods.

cystine, mixed disulfides such as cysteinyl glutathione, or cysteine sulfenic acid. Other S-nitrosothiols may similarly produce reactive species that play a role in the observed biological responses.

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