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Critical Role of Sulfenic Acid Formation of Thiols in the Inactivation of Glyceraldehyde-3-phosphate Dehydrogenase by Nitric Oxide

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ABSTRACT. The relationship between possible modifications of the thiol groups of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by nitric oxide (NO) and modified enzyme activity was examined. There are 16 free thiols, including 4 active site thiols, in a tetramer of GAPDH molecule. NO donors, sodium nitroprusside (SNP), and *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) decreased the number of free thiols with a concomitant inhibition of GAPDH activity in a concentration- and time-dependent manner. After treatment for 30 min, free thiols were maximally decreased to 8–10 per GAPDH tetramer and enzyme activity was also inhibited to 5–10% of control activity. In the presence of 30 mM dithiothreitol (DTT), these effects were completely blocked. Since similar results were obtained in the case of hydrogen peroxide (H_2O_2) treatment, which is known to oxidize the thiols, these effects of nitric oxide donors were probably due to modification of thiol groups present in a GAPDH molecule. On the other hand, DTT posttreatment after the treatment of GAPDH with SNP, SNAP, or H_2O_2 did not completely restore the modified thiols and the inhibited enzyme activity. DTT posttreatment after the 30-min-treatment with these agents restored free thiols to 14 in all treatments. In the case of SNAP treatment, all 4 active sites were restored and enzyme activity reached more than 80% of the control activity, but in two other cases one active site remained modified and enzyme activity was restored to about only 20%. Therefore, all 4 free thiols in the active site seem to be very important for full enzyme activity. DTT posttreatment in the presence of sodium arsenite, which is known to reduce sulfenic acid to thiol, almost completely restored both thiol groups and enzyme activity. These findings suggest that nitric oxide inhibits GAPDH activity by modifications of the thiols which are essential for this activity, and that the modification includes formation of sulfenic acid, which is not restored by DTT. *S*-nitrosylation, which is one type of thiol modification by NO, occurred when GAPDH was treated with SNAP but not SNP. Analysis of thiol modification showed that SNAP preferentially nitrosylated the active site thiols, the nitrosylation of which fully disappeared by DTT posttreatment. It seems that SNAP nitrosylates the active site thiols of GAPDH to prevent these thiols from oxidizing to sulfenic acid. BIOCHEM PHARMACOL **58**;1:133–143, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. thiol oxidation; GAPDH; nitric oxide, sulfenic acid; SNAP; SNP

NO§ mediates multiple functions, including smooth muscle relaxation, neurotransmission, and macrophage-mediated cytotoxicity [1, 2]. Most of its molecular actions are explained by activation of soluble guanylate cyclase, leading to cGMP formation and protein phosphorylation via cGMP-dependent protein kinase [3]. However, many recent studies indicate that NO inhibits GAPDH, a glycolytic enzyme, in a cGMP-independent fashion [4, 5]. GAPDH functions in glycolysis, where it catalyzes the oxidative phosphorylation of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate. The inhibition of GAPDH leads to suppression of glycolysis and depletion of intracellular ATP, and finally results in cell death. Thus, the effect of NO on GAPDH activity seems to be associated with the mechanisms responsible for NO-mediated cytotoxicity and pathological responses [6–8].

Several studies on the NO-mediated inactivation of GAPDH have suggested that treatment of GAPDH with NO gas or NO donors such as SNP leads to ADP ribosylation [9–11] and/or covalent binding of NAD^+ to active site thiol of the enzyme [12]. However, the stoichiometry of NAD^+ incorporation into the GAPDH molecule was quite

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§ *Abbreviations:* SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; SNP, sodium nitroprusside; NO, nitric oxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DTT, dithiothreitol; GSNO, *S*-nitrosoglutathione; H_2O_2, hydrogen peroxide; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); and NEM, *N*-ethylmaleimide.

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low even under the conditions of complete inhibition of the enzyme [12, 13]. Therefore, the NAD^+-dependent modification does not appear to be the sole mechanism responsible for NO-mediated inhibition of the enzyme. On the other hand, it has been reported that treatment of GAPDH with NO gas or *S*-nitroso compounds such as GSNO leads to *S*-nitrosylation of the enzyme, the modification of which was correlated with NO-mediated inactivation of the enzyme [13–15]. More recently, it was suggested that NADH rather than NAD^+ was a preferred substrate for covalent modification by NO, and that *S*-nitrosylation and NADH attachment were responsible for reversible and irreversible enzyme inhibition, respectively [16]. However, it remains unknown how *S*-nitrosylation is implicated in the actual sequential reaction of NO-mediated inhibitory mechanisms.

An excess amount of thiol reagent such as DTT has been known to protect GAPDH from NO-mediated inactivation [12, 13]. This result implies that oxidation of thiol groups of GAPDH molecule might be involved in the NO-mediated inactivation of the enzyme, since NO is extremely susceptible to oxidation to form higher oxidation states of nitrogen oxides such as NO_2 and N_2O_3 under aerobic conditions.

In this study, we planned a series of experiments to characterize the thiol modifications in which the enzyme was inactivated to a different degree, and carefully analyzed the relationship between thiol modification and enzyme inactivation with three different reagents, SNP and SNAP as NO donors, and H_2O_2 as oxidant. Our results indicate that the oxidation of thiol to sulfenic acid is critical for the NO-mediated inactivation of GAPDH. In addition, SNAP was suggested to protect active thiols from further oxidation such as sulfenic acid formation.

MATERIALS AND METHODS

Materials

Sephadex G-25 (fine) was purchased from Pharmacia Biotech. NAD^+ was from Boehringer Mannheim. GAP and ATP were from Sigma Chemical Co. SNP and SNAP were from Doujin Laboratories. [adenylate-^{32}P]NAD^+ was from Dupont New England Nuclear. All other chemicals were of analytical grade and were purchased from either Sigma or Wako Pure Chemical Co.

Preparation of GAPDH

Purified rabbit muscle GAPDH was obtained from Boehringer Mannheim, which was supplied as a 10 mg/mL suspension in 3.2 M $(NH_4)_2SO_4$. Just before use, a sample was centrifuged (14,000 *g*, 10 min), the supernatant was removed, and the pellet was suspended at ~5 mg/mL in 0.5 M HEPES buffer (pH 7.6) containing 5 mM EDTA. Protein concentration was estimated by the method of Bradford using BSA as a standard.

Treatment of GAPDH with NO Donor

GAPDH was treated with various concentrations of NO donors such as SNP and SNAP, or of a oxidizing agent, H_2O_2, in the presence of 1 mM EDTA and 1 μM NAD^+ in 50 mM HEPES buffer (pH 8.5), at 30° for varying periods of time. After incubation with these inhibitors, the reaction was terminated by passing through a spin column (800 *g* for 3 min) filled with Sephadex G-25 equilibrated with 50 mM HEPES buffer (pH 8.5) containing 1 mM EDTA to remove the inhibitors, and the sample was subjected to the following experiments.

GAPDH Activity

GAPDH activity was determined by the method of Willson *et. al.* [17], with some modifications. The reaction mixture (1 mL) was 100 mM triethanolamine hydrochloride buffer (pH 7.6) containing 1 mM EDTA, 0.1 M KCl, 10 mM K_2HPO_4, 0.2 mM NAD^+, and 0.8 mM D-glyceraldehyde 3-phosphate. The reaction was started by the addition of GAPDH (~5 μg) to the reaction mixture at room temperature. The initial velocity of an increase in absorbance at 340 nm due to the formation of NADH was measured for 2 min using a Hitachi U-2000A spectrophotometer.

Titration of Thiols

Free thiols in GAPDH were titrated by the method with DTNB as described by Ellman [18]. The enzyme samples (0.1 mg/mL) were incubated with 0.1 mM DTNB in 100 mM potassium phosphate buffer (pH 7.6). The absorbance at 412 nm was successively recorded for 15 min at 25° in the presence of 6 M urea to unmask masked thiols. Active site thiols were determined from the time-course of the reaction between free thiols and DTNB. The time-course was biphasic; four thiols per tetramer, which correspond to the active site Cys149 of each monomer, reacted very quickly (first phase), and the remaining 12 thiols reacted slowly (second phase) [19]. A molar extinction coefficient (ε412 nm) of 13,600 M^{-1} cm^{-1} for the anion of thionitrobenzoic acid was used to determine the number of titrated thiols.

Quantitative Determination of S-Nitrosothiols

The *S*-nitrosothiols of GAPDH were determined as described by Saville [20]. *S*-nitrosylated enzyme was incubated with 10 mM mercuric chloride for 15 min at room temperature to hydrolyze the *S*-nitrosobond. The resultant nitrite was determined using the Griess reaction as follows. Briefly, after the treatment with mercuric chloride, the sample was centrifuged at 16,000 *g* for 5 min, and the supernatant was added to an equal volume of Griess reagent (0.6% sulfanilamide, 0.06% naphthalene-ethylene diamine dihydrochloride in 6% H_3PO_4) and immediately mixed. After incubation for 5 min at room temperature, the absorbance of the

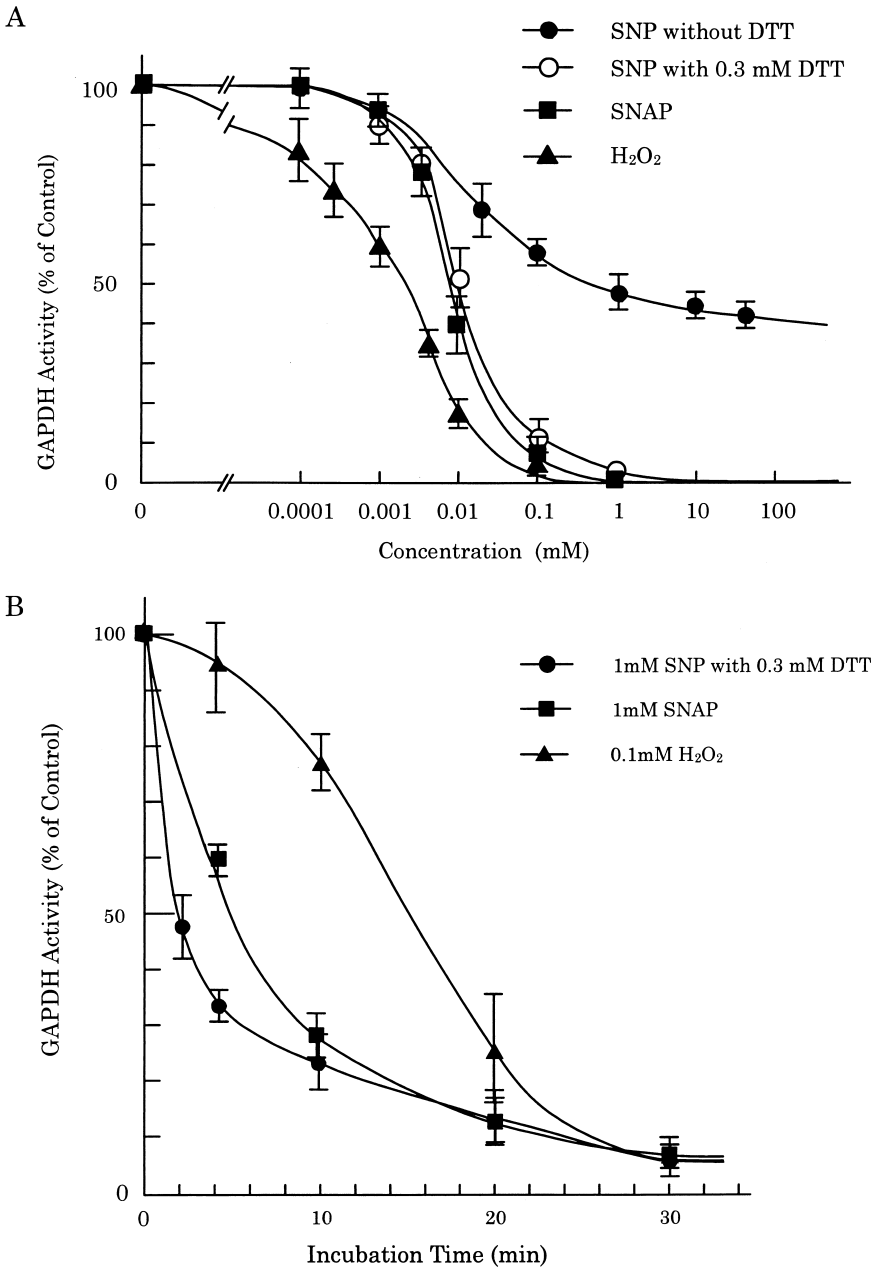

FIG. 1. Concentration-(A) and time-(B) dependent inhibition of GAPDH activity by SNP, SNAP, or H_2O_2. (A) Purified rabbit muscle GAPDH at a concentration of 50 mg/mL was treated with indicated concentrations of SNAP, H_2O_2, or SNP in the presence or absence of 0.3 mM DTT for 30 min at 30°. (B) GAPDH at a concentration of 50 mg/mL was treated with 1 mM SNAP, 100 μM H_2O_2, or 1 mM SNP with 0.3 mM DTT at 30° for the various incubation times indicated. The reaction was stopped by passing the reaction mixture through a Sephadex G-25 column to remove the agents. The eluate was used for determination of GAPDH activity as described in Materials and Methods. GAPDH activity is expressed as a percentage of the control. Values are the means ± SD of three separate experiments.

reaction product (diazotization of nitrite) was read at 548 nm. A molar extinction coefficient (ε548 nm) of 44,000 M^{-1} cm^{-1} for the diazotization of nitrite was used to determine the number of *S*-nitrosothiols.

RESULTS

Effects of SNAP, SNP, and H_2O_2 on GAPDH Activity

SNAP and SNP inhibited GAPDH activity in a concentration-dependent manner. H_2O_2, an oxidizing agent, also inhibited GAPDH activity (Fig. 1). H_2O_2 is known to inhibit enzyme activity by oxidizing thiols in GAPDH [21, 22]. When the incubation was carried out for 30 min to obtain the maximal inhibition, the concentrations for the half-maximal inhibition (IC_{50}) of SNAP, SNP, and H_2O_2 were 9, 12, and 3 μM, respectively. SNP was always used in the presence of 0.3 mM DTT, because DTT accelerates the release of NO from SNP molecule [13]. Indeed, SNP in the absence of DTT did not show sufficient inhibition in comparison to that in its presence (Fig. 1A). In all the following experiments, the enzyme was treated with 1 mM SNAP, 1 mM SNP (in the presence of 0.3 mM DTT), or 0.1 mM H_2O_2.

Relationship between Enzyme Activity and the Number of Free Thiols after Treatment of GAPDH with SNAP, SNP, or H_2O_2

Half-maximal inhibition of GAPDH occurred 5, 2, and 15 min after treatment with 1 mM SNAP, 1 mM SNP (in the presence of 0.3 mM DTT), and 0.1 mM H_2O_2, respectively (Fig. 1B). Both SNAP and SNP rapidly inhibited the

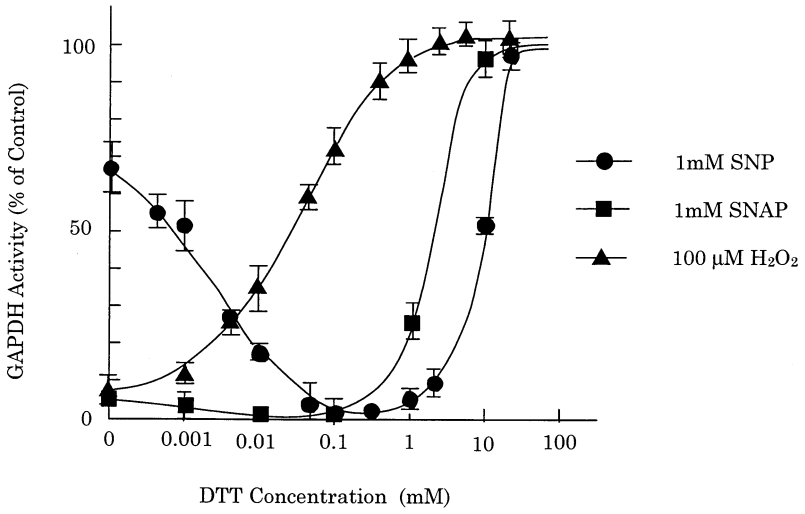

FIG. 2. Effect of DTT on SNAP-, SNP-, and H_2O_2-induced inhibition of GAPDH activity. GAPDH was treated with 1 mM SNAP, 1 mM SNP, or 100 μM H_2O_2 in the absence or presence of DTT at the indicated concentrations for 30 min at 30° in 50 mM HEPES (pH 8.5) containing 10 μM NAD^+ and 1 mM EDTA. The reaction was stopped by passing the reaction through a Sephadex G-25 column to remove the agents and the eluate was used for determination of GAPDH activity as described in Materials and Methods. GAPDH activity is expressed as a percentage of the control. Values are the means ± SD of three separate experiments.

enzyme activity in comparison with H_2O_2. We next measured free thiols before and after treatment of GAPDH with SNAP, SNP, or H_2O_2. GAPDH is composed of 4 identical subunits and each monomer contains 4 thiols, making 16 thiols in all [23]. All sixteen thiols per tetramer were detected in the untreated GAPDH. Treatment with 1 mM SNAP, 1 mM SNP (in the presence of 0.3 mM DTT), or 0.1 mM H_2O_2 for 30 min resulted in a decrease in the number of free thiols to 8, 10, or 11, respectively (Fig. 3A). The presence of 20 mM DTT during treatment completely protected the enzyme activity from inhibition (Fig. 2) and the thiols from modification (data not shown). The results suggest that modification of thiols is involved in the mechanism of enzyme inhibition by these agents.

Time-courses of thiol modification by both SNP and H_2O_2 were single step (t½; 1 min for SNP, 8 min for H_2O_2) (Fig. 3A), whereas modification by SNAP was two steps (t½; 3 min for the fast phase, 26 min for the late phase). These results suggest that SNAP modifies thiol groups included in a GAPDH molecule via two different mechanisms.

Modification of Active Site Thiols by SNAP, SNP, and H_2O_2

GAPDH activity is known to be sensitive to thiol modification, because Cys149 is present in the active site of the enzyme [21, 22, 24, 25]. Therefore, we also examined whether active site Cys149 is modified by these agents. It was found that SNAP modified two thiols but that SNP and H_2O_2 modified only one of the four active site thiols, and that modification of active site thiols by SNAP and SNP rapidly occurred within 2 min after addition of the agents (Fig. 3B).

S-Nitrosylation of GAPDH Induced by SNAP

It has been reported that *S*-nitrosoglutathione reversibly inhibits GAPDH activity via *S*-nitrosylation, which is one

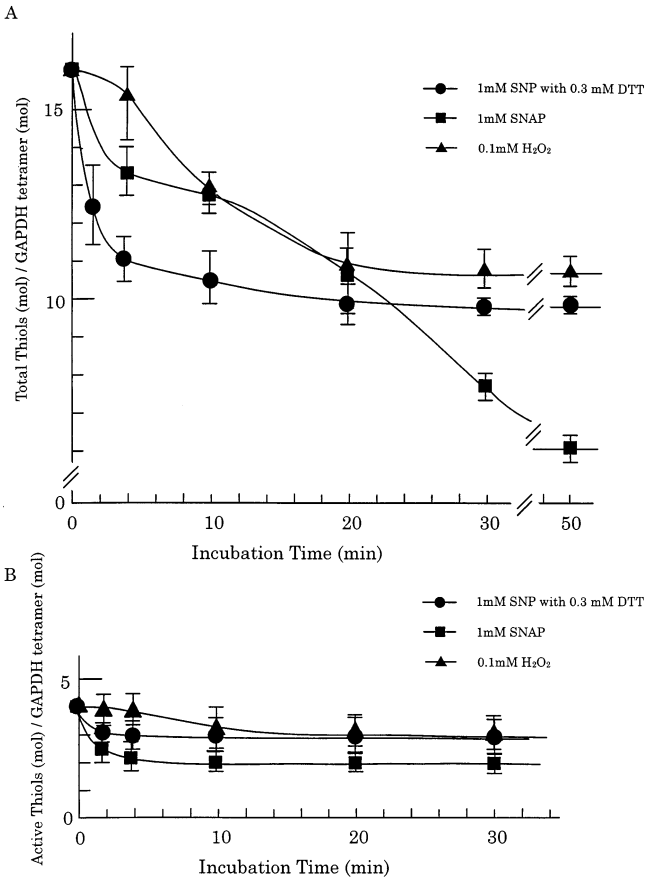

FIG. 3. Time-course of thiol modification of GAPDH by treatment of GAPDH with SNAP, SNP, or H_2O_2. GAPDH was treated with 1 mM SNAP, 1 mM SNP (in the presence of 0.3 mM DTT), or 100 μM H_2O_2 for the various times indicated, then the reaction was stopped by passing the reaction mixture through a Sephadex G-25 column to remove the inhibitors. The eluate was used for determination of the thiol groups as described in Materials and Methods. The number of total free thiols (A) and active sites (B) were determined by the method described by Ellman [18] and expressed as the number of thiols/GAPDH tetramer. Values are expressed as the means ± SD of four separate experiments.

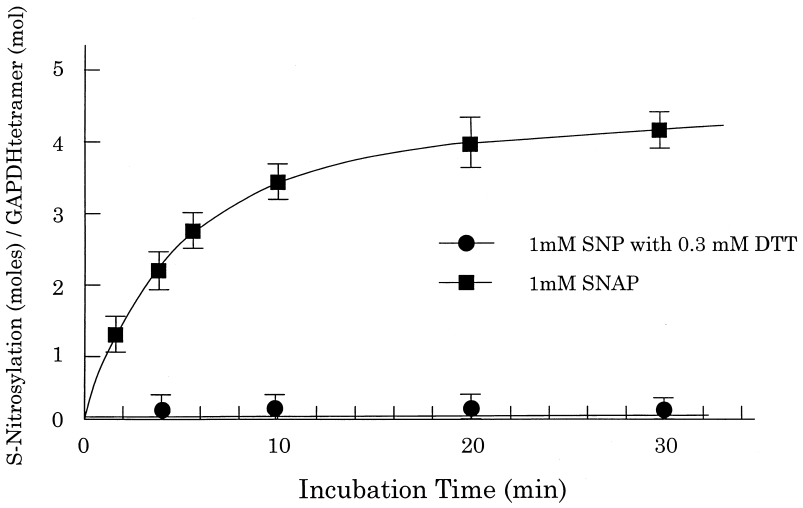

FIG. 4. Time-course of the formation of S-nitrosylation by treatment with SNAP or SNP. GAPDH was treated with 1 mM SNAP or 1 mM SNP (in the presence of 0.3 mM DTT) for the times indicated, then the reaction was stopped by passing the reaction mixture through a Sephadex G-25 column to remove the agents. The eluate was used for determination of the number of S-nitrosothiols as described in Materials and Methods. Values are number of S-nitrosylations/GAPDH tetramer and expressed as the means ± SD of four separate experiments.

of the thiol modifications induced by NO [13, 14]. Therefore, we further examined whether SNAP and SNP *S*-nitrosylate the thiols of GAPDH. As shown in Fig. 4, SNAP *S*-nitrosylated 4 thiols/tetramer of GAPDH during 30-min incubation, but SNP did not. The time-course of *S*-nitrosylation induced by SNAP (Fig. 4) was similar to the first phase in that of thiol modification (Fig. 3A), suggesting that the fast phase of thiol modification results from the *S*-nitrosylation (Figs. 3A and 4). In the fast phase of thiol modification, two active site thiols of GAPDH were *S*-nitrosylated within 4 min after SNAP treatment.

Effects of DTT Posttreatment on GAPDH Inactivated by and on Thiols Modified by SNAP, SNP, or H_2O_2 Treatment

Since the presence of DTT during the treatment of GAPDH with SNAP, SNP, or H_2O_2 resulted in complete protection of the enzyme from inactivation and of the thiols from modification induced by the agents (Fig. 2), we next examined whether a posttreatment with DTT after the SNAP, SNP, or H_2O_2 treatment restores GAPDH from the inactivation and modification. The enzyme was first treated with these agents for various incubation periods up to 30 min. After the agents were rapidly removed, the inactivated GAPDH was posttreated with DTT. In the case of the short treatment with the agents, the inactivated GAPDH was fully reactivated with a concomitant recovery of thiols from the modified state. However, the recovery of the enzyme from inhibition and thiols from modification was significantly weakened by the long treatment, even though the extent of recovery was different among those agents (Fig. 5). The results suggest that there are two types of inactivation of GAPDH by NO donors or H_2O_2, that is either restored or not restored by DTT posttreatment, and that the former seems to be associated with formation of disulfide by the agents as well as with *S*-nitrosylation in the case of SNAP.

Treatment of GAPDH with SNAP, SNP, or H_2O_2 for 30 min resulted in an almost complete inhibition of enzyme activity. In this case, the DTT posttreatment only partially restored the enzyme activity inhibited by both SNP and H_2O_2 treatment, but drastically recovered SNAP-induced GAPDH inhibition to 90% of control activity. However, the number of thiols restored was 14 in all these reagents (Fig. 5 and Table 1). Thus, the extent of enzyme inactivation is not necessarily related to the number of thiol groups modified. The site of thiols present in the GAPDH molecule may be important, that is, active site thiols or other thiols than active site.

Effects of DTT Posttreatment on Active Site Thiols Modified by SNAP, SNP, or H_2O_2 Treatment

Treatment of GAPDH with SNAP, SNP, and H_2O_2 for 30 min reduced the free thiols to 8, 10, and 11, respectively and active site thiols to 2, 3, and 3, respectively (Table 1). DTT posttreatment restored the number of thiols to 14 in all cases, but two thiols remained modified. The active site thiols were fully restored in SNAP treatment, but not in SNP or H_2O_2 treatment (Table 1). Thus, SNP and H_2O_2 induced modification of the active site Cys149 which was not restored by DTT posttreatment. It should be noted that two active site thiols in SNAP treatment were preferentially nitrosylated. The thiols nitrosylated by SNAP were fully restored by the treatment with 20 mM DTT (Fig. 6, inset). These results suggest that *S*-nitrosylation induced by SNAP protects the two active site thiols from the modification which is not restored by DTT posttreatment.

Effects of Sodium Arsenite on GAPDH Inactivated by and on Thiols Modified by SNAP, SNP, or H_2O_2 Treatment

DTT can restore thiols from modifications such as disulfide and *S*-nitrosylation (Fig. 6, inset; see also 21, 26). On the other hand, sodium arsenite is known to reduce sulfenic acid but not to reduce disulfide to thiol [21, 25, 26].

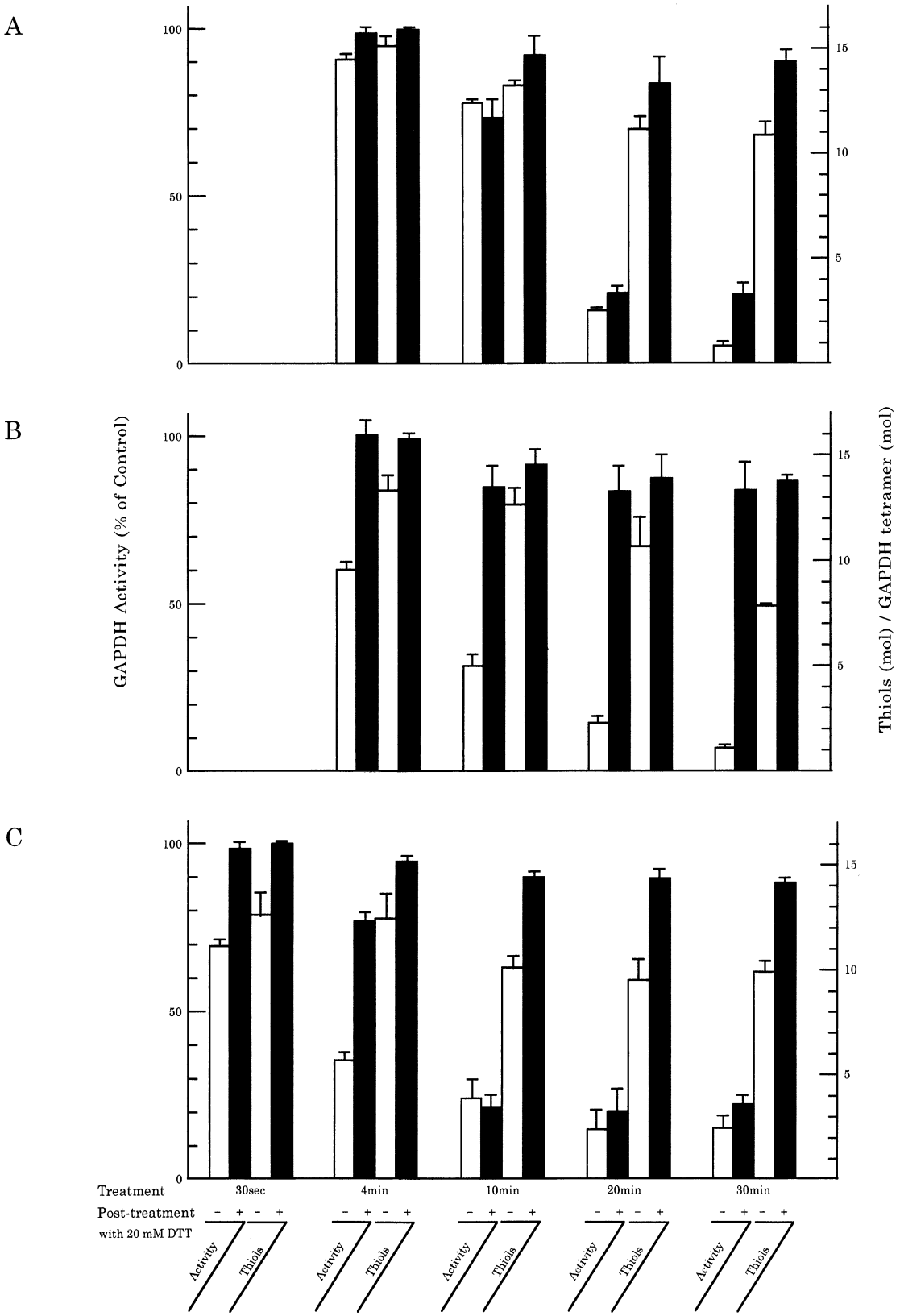
A
B
C
GAPDH Activity (% of Control)
Thiols (mol) / GAPDH tetramer (mol)
100
50
0
15
10
5
Treatment
30sec
4min
10min
20min
30min
Post-treatment
with 20 mM DTT
- + - +
Activity
Thiols

FIG. 5. Restoration by DTT posttreatment of GAPDH activity inactivated by SNP (A), SNAP (B), and H_2O_2 (C). GAPDH was first treated with 1 mM SNAP, 1 mM SNP (in the presence of 0.3 mM DTT), or 100 μM H_2O_2 for the indicated times, then the reaction was stopped by passing the reaction mixture through a Sephadex G-25 column to remove the agents. A portion of the eluate was used for determination of GAPDH activity and titration of free thiols. The remaining eluate was further incubated in the presence (+) or absence (−) of 20 mM DTT for 30 min, and the sample was passed through a Sephadex G-25 column to remove excess DTT. The sample was concentrated by filtration using Ultrafree-MC filters (Millipore) and subjected to determination of GAPDH activity and titration of free thiols as described in Materials and Methods. GAPDH activity is expressed as a percentage of the control. Values are the means ± SD of five separate experiments.

Therefore, it is of interest to study the effect of sodium arsenite on thiol modification which is not restored by DTT posttreatment. As shown in Fig. 6, posttreatment with sodium arsenite restored two thiols from modification in all cases of SNAP, SNP, and H_2O_2 treatment, suggesting that these two thiols had been oxidized to sulfenic acid by these reagents. Under the same conditions, however, enzyme activity was restored only to <20% or not restored at all in SNAP-induced or SNP- and H_2O_2-induced inactivation, respectively (Fig. 6). Thus, posttreatment with either DTT or sodium arsenite separately could not fully restore thiol groups nor enzyme activity. However, the combination of DTT and sodium arsenite fully restored both enzyme activity and thiol groups in GAPDH which had been inactivated by all these agents (Fig. 6, A, B and C). Thus, the modification of two active site thiols to sulfenic acid also seems to be an important mechanism in inhibition of GAPDH induced by NO or NO donors.

DISCUSSION

In the present work, we studied the thiol modification of GAPDH by NO and characterized the property of *S*-nitrosylation involved in the mechanisms of NO-mediated inactivation of the enzyme. We found that the NO-mediated thiol modification not restored by DTT posttreatment is that of oxidation of thiol to sulfenic acid, and that SNAP preferentially nitrosylated the active site thiols. It was suggested that *S*-nitrosylation by SNAP prevents active site thiols from forming sulfenic acid. We used relatively high concentrations of SNP and SNAP in an attempt to determine the maximum effects of NO donors on both enzyme activity and thiol modification, and to study their restoration by DTT posttreatment. However, the IC_{50} values of these NO donors are relatively low (9 μM for SNAP and 12 μM for SNP, in Fig. 1). Therefore, the present results might provide substantial information as to the mechanisms of NO action on GAPDH activity *in vivo*.

TABLE 1. Thiol modification of GAPDH by SNAP, SNP, or H_2O_2 and restoration from the modified thiol to free thiol by DTT posttreatment

	Thiols(mol)/GAPDH tetramer(mol)			
	Posttreatment with 0 mM DTT		Posttreatment with 20 mM DTT	
Reagent	Active site	Total	Active site	Total
1 mM SNAP	2.1 ± 0.1	7.8 ± 0.4	4.2 ± 0.1	13.8 ± 0.2
1 mM SNP	2.8 ± 0.4	9.9 ± 0.8	2.9 ± 0.1	14.0 ± 0.1
100 μM H_2O_2	3.0 ± 0.3	11.0 ± 0.4	3.0 ± 0.1	14.1 ± 0.4

GAPDH was first treated with 1 mM SNAP, 1 mM SNP (containing 0.3 mM DTT), or 100 μM H_2O_2 at 30° for 30 min, and then the reaction was terminated by passing over a Sephadex G-25 column to remove the inhibitors. After a 30-min interval, the sample was further incubated at 30° for 30 min with or without 20 mM DTT and subjected to the Sephadex G-25 column to remove excess DTT. Determination of the number of free thiols was performed as described in Materials and Methods. The number of free thiols of active (Cys149) and of total sites/GAPDH tetramer is expressed as the mean ± SD of six separate experiments.

NAD^+-dependent Modification

Many studies have shown that treatment of GAPDH with NO gas and NO donors leads to an NAD^+-dependent modification [5, 12, 14, 27]. However, the stoichiometry of NAD^+ incorporation was quite low even under the conditions of complete inhibition of the enzyme. Therefore, NAD^+-dependent modification does not appear to be a main mechanism for NO-mediated inhibition of the enzyme [12, 13]. Indeed, in our study, NAD^+ incorporation into the GAPDH monomer measured with [^{32}P]NAD was only ~0.8% of the enzyme molecule when maximal NAD^+-dependent modification was induced by treatment with SNP or SNAP (data not shown). The treatment with a combination of DTT and sodium arsenite significantly, but not entirely, restored enzyme activity up to ~97% from inhibition by SNAP and SNP (Fig. 6). It seems likely that NAD^+-dependent modification occurs in a few molecules of the enzyme and remains even after treatment with a combination of DTT and sodium arsenite. Thus, NAD^+-dependent modification does not seem to be important in GAPDH inactivation mediated by the NO donors.

Involvement of Two Different Thiol Oxidation States in NO-mediated GAPDH Inactivation

We studied the properties of thiol modification by NO in comparison with those by H_2O_2 to ascertain the relationship between the thiol oxidation and NO-mediated inactivation of GAPDH, because it is reasonable to postulate that NO gas and NO donor under aerobic conditions generate higher oxidation states of nitrogen oxides such as NO_2 and N_2O_3 and in turn that the nitrogen oxides oxidize thiols [28]. An excess of DTT added together with NO donors protected GAPDH from inactivation by NO donors and H_2O_2. DTT posttreatment after NO donor treatment for a short period also fully restored the GAPDH activity from inactivation, with concomitant restoration from the

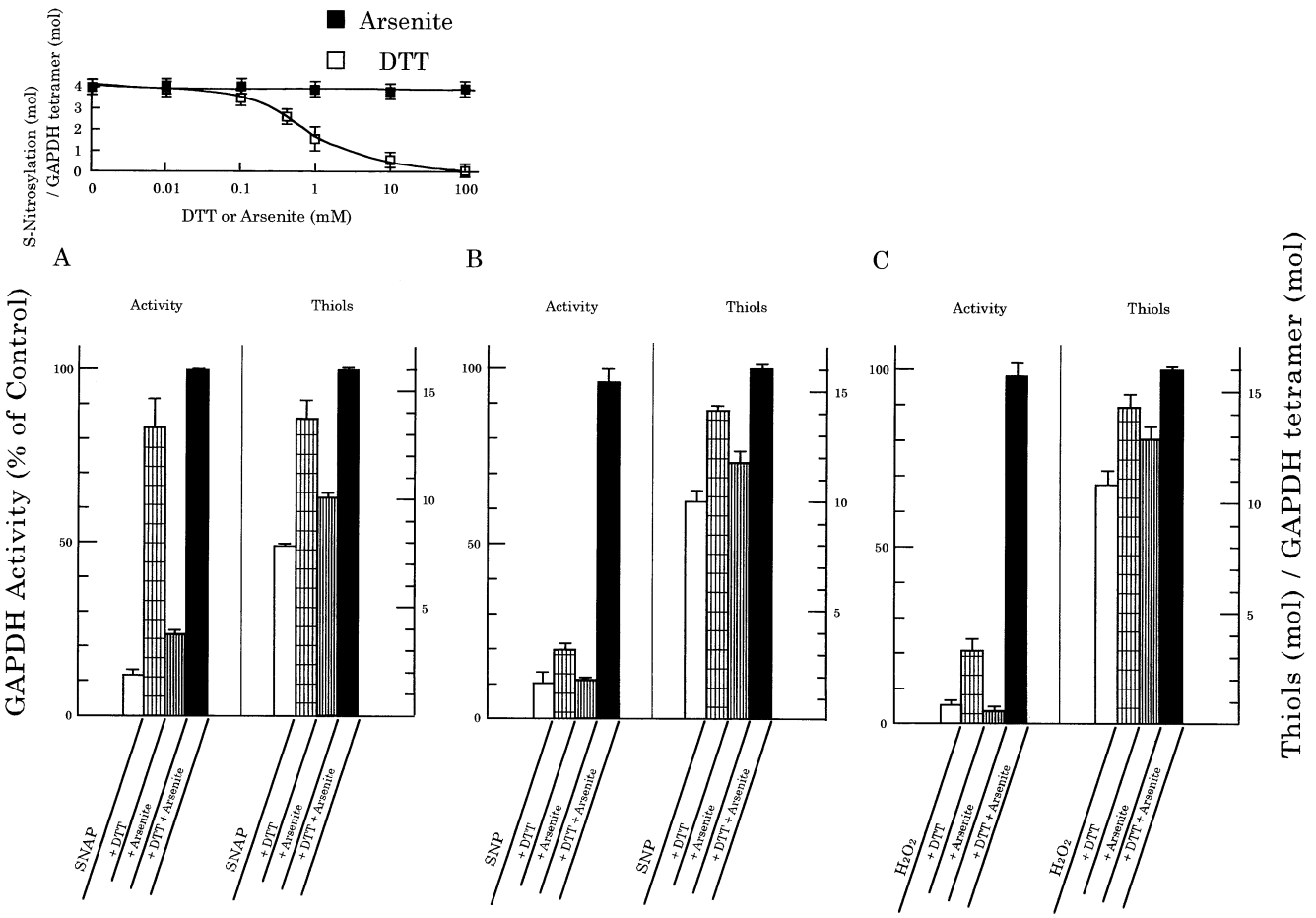

FIG. 6. Restoration by DTT posttreatment without or with arsenite of inactivated GAPDH and of modified thiols by SNAP (A), SNP (B), or H_2O_2 (C) treatment. GAPDH was first treated with 1 mM SNAP, 1 mM SNP (in the presence of 0.3 mM DTT), or 100 μM H_2O_2 for 30 min, then the reaction was stopped by passing the reaction mixture through a Sephadex G-25 column to remove the agents. A portion of the eluate was used for determination of GAPDH activity and titration of free thiols. The remaining eluate was further incubated with 20 mM DTT alone, 25 mM sodium arsenite alone, or the two together for 30 min, and the sample was passed through a Sephadex G-25 column to remove excess DTT and sodium arsenite. The sample was concentrated by filtration using Ultrafree-MC filters (Millipore) and subjected to determination of GAPDH activity and titration of free thiols as described in Materials and Methods. The inset shows the effect of posttreatment with DTT and sodium arsenite on S-nitrosylation of GAPDH induced by SNAP. Values are the means ± SD of five separate experiments.

modified thiols. However, the long NO donor treatment significantly weakened restoration from both inactivation and modification. Thus, two types of thiol modification, i.e. restored and not restored by DTT posttreatment, were involved in NO- and H_2O_2-mediated GAPDH inactivation. In the present study, treatment with a combination of DTT and sodium arsenite almost fully restored both enzyme activity and thiol groups in all cases of SNAP, SNP, and H_2O_2 treatment, although treatment with either DTT or sodium arsenite alone could not restore the thiols or activity (Fig. 6). These results suggest that two different thiol oxidations, formation of disulfide and sulfenic acid, are involved in NO-mediated GAPDH inactivation.

Possible Contribution of S-Nitrosylation to the Protection of Active Site Thiols from Oxidation

In all cases of SNP-, SNAP-, and H_2O_2-mediated inactivation of GAPDH, DTT posttreatment restored free thiols up to 14 thiol groups/tetramer. The remaining 2 thiols seemed to be oxidized to sulfenic acid (Table 1). Under the same conditions, enzyme activity was restored up to 90% of control activity in SNAP-mediated inactivation, but only partially in both SNP- and H_2O_2-mediated inactivation (Figs. 5 and 6). The same results were also obtained after treatment with GSH instead of DTT (data not shown). Thus, the level of enzyme restoration by DTT posttreatment differed among the agents used for treatment, although the number of thiols restored was rather constant. GAPDH consists of homotetramer whose monomer contains four thiols, one of which (Cys149) locates in the active site critical for catalysis of the enzyme and is extremely sensitive to thiol modification. Indeed, this active site cysteine has been reported to be the target for several oxidants [21, 22, 24–26]. Although two of the four Cys149s in the tetramer indeed participate in the binding of NAD^+ to the active site, the modification of any of these

four thiols causes it to lose its catalytic activity [29]. We suggested that both SNP and H_2O_2 oxidize only one of the four active site thiols to sulfenic acid, which is not reduced by DTT, and that SNAP modifies two of the four active site thiols to *S*-nitrosylation, these being fully restored by DTT posttreatment (Figs. 3, 4, and 6 [inset]). Thus, restoration from enzyme inactivation seems to be more closely related to restoration from thiol modification at the active sites than at other sites. These results suggest that SNAP protects the active site thiols from oxidation by *S*-nitrosylation. Although the reason for the inability of SNP to *S*-nitrosylate remains unknown, the different efficiency of NO donors to *S*-nitrosylate may depend on the different mechanisms of NO release from the donors.

Reactivity of the Active Site Thiols

It was shown that modification of active site thiols by both SNAP and SNP occurred more rapidly than modification by H_2O_2 (Fig. 3B), suggesting that the active site thiols are more sensitive to NO than oxidants. The 30-min treatment with SNP and H_2O_2 modified 6 and 5 thiols, respectively, but only one of four active site thiols was modified in both treatments (Fig. 3). On the other hand, SNAP modified 8 thiols for the same period, two of which were active site thiols. Thus, all of the active site thiols were not modified by these agents, even though these thiols were believed to be highly subject to thiol modification. GAPDH molecule is tetrameric and binds four molecules of the coenzyme, NAD^+, at the four active sites. After treatment of GAPDH with various agents modifying Cys149 [30] or with substrate analogue β-(2-furyl)-acryloyl phosphate [31], two active sites were preserved. Thus, two of the four active sites seem to be less sensitive to thiol-blocking agents. The tetramer exhibits negative cooperativity in the binding of NAD^+: the first two molecules of the coenzyme are tightly bound, the other two loosely bound [23–34]. Birkett [35] has demonstrated that an increased concentration of NAD^+ protected the active site thiols from modification by thiol-blocking agents such as ethacrynic acid, suggesting that tight binding of NAD^+ protects the active site thiols from modification. Therefore, the two less sensitive thiols of the four active sites may bind NAD^+ tightly. This might be the reason why we did not detect the modification of three or four active site thiols after treatment with SNAP, SNP, or H_2O_2 in the present study.

Possible Importance of Cys149 and Cys153 in Restoration of Enzyme Activity

DTT posttreatment restored 14 thiols in all cases of SNAP, SNP, and H_2O_2 treatment, but two thiols which were oxidized to sulfenic acid remained modified. In this state of GAPDH, SNAP-induced enzyme inhibition was restored up to 90% of control activity, but SNP- and H_2O_2-induced inhibition was only partially restored (Fig. 5), and active site thiols were fully restored in SNAP treatment but not in SNP or H_2O_2 treatment (Table 1). Thus, the restoration of active site thiols seems critical for enzyme activity. However, although the arsenite posttreatment restored all of the active site thiols in both SNP and H_2O_2 treatment, the enzyme activity was not restored (Fig. 6). These results suggest that other thiols than active site Cys149s are also important for enzyme activity.

Based on analysis of the reaction of thiols with DTNB, the 16 thiols can be classified into three groups: 4 thiols reacting very quickly with DTNB (which correspond to the active site thiols), 4 reacting at an intermediate rate, and 8 reacting slowly [35]. It has been reported that one of the thiol-blocking agents, ethacrynic acid selectively abolished the reaction of the first two groups with DTNB [35]. Thus, not only active site thiols but also those which react with DTNB at the intermediate rate are sensitive to oxidizing agents. In the present study, treatment of GAPDH with SNAP, SNP, and H_2O_2 abolished the fast reaction with DTNB of 2, 1, and 1 thiol/tetramer, respectively. However, we could not clarify whether the other thiols abolished were those reacting intermediately or slowly with DTNB. Wassarman and Major [36] demonstrated in lobster muscle GAPDH that reaction of the active site Cys148 (Cys149 in the case of rabbit muscle GAPDH) with DTNB was followed by formation of an intramolecular disulfide bond between Cys148 and Cys152 (Cys153 in the case of rabbit muscle GAPDH). Moreover, it has been reported that various oxidizing agents reversibly inactivated GAPDH from yeast [37] and pig muscle [38] by forming the intramolecular disulfide bond between these thiols. Thus, in addition to active site Cys149s, Cys153s are most likely sensitive to oxidizing agents. Comparative studies on the primary structures of GAPDH from different species of animals have shown that the amino acid sequence around the active site cysteine residue is highly conservative over a range of 12 amino acids [39, 40]. This invariant sequence is Ser-Asn-Ala-Ser-Cys*149-Thr-Thr-Asn-Cys153-Leu-Ala-Pro- (The active site cysteine is marked with an asterisk). Cys153 is very close to the active site Cys149 and is also one of the evolutionally conservative amino acids. Thus, Cys153 also seems to be important for enzyme activity. If such is the case, DTT or sodium arsenite posttreatment after SNP and H_2O_2 treatment might restore either Cys149 or Cys153, but DTT posttreatment after SNAP treatment might restore both Cys149 and Cys153, resulting in a significant restoration in enzyme activity.

Recent studies on cytotoxicity mediated by oxidants have shown that NO and nitroso compounds such as SNAP and GSNO, but not SNP, protected the cells from injury mediated by H_2O_2 [41–43] and brain dopamine neurons from oxidation stress induced by iron *in vivo* [44, 45]. In these studies, the authors suggested that NO inhibits iron- and/or H_2O_2-mediated oxidant generation and lipid peroxidation. Interestingly, GSNO has been identified in cells and tissues containing NO synthase and proposed as an endogenous NO reservoir [46]. Rauhala *et al.* [45] found that an antioxidative effect of GSNO is more potent than

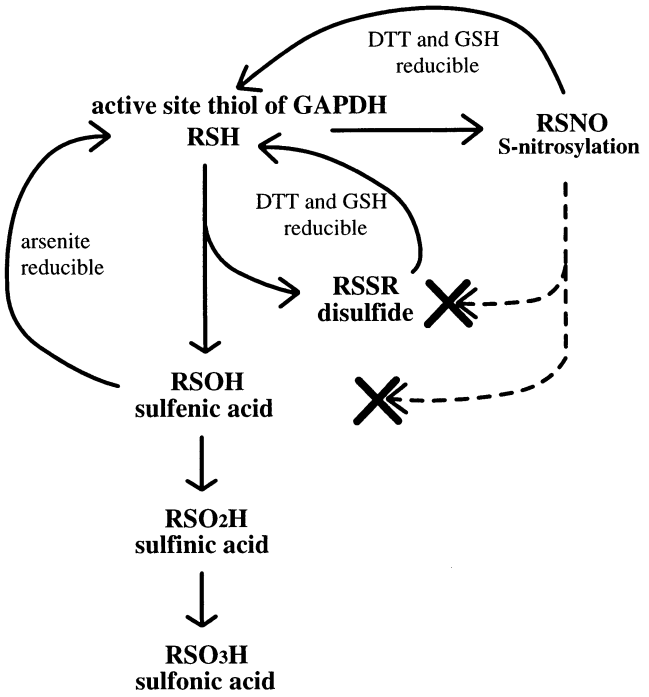

FIG. 7. Protection of the active site thiol of GAPDH from oxidation via *S*-nitrosylation. NO preferentially *S*-nitrosylates active site thiol and protects it from oxidation (X). The *S*-nitrosylated thiol and disulfide, but not sulfenic acid, are reversibly restored to thiol by DTT or GSH. Arsenite reduces sulfenic acid to thiol only *in vitro*. Sulfenic acid is further oxidized to the stable higher oxidation products sulfinic acid or sulfonic acid.

that of its precursor GSH, suggesting that GSNO may play an important role in the cellular antioxidative defense system. Thus, NO might serve a defensive function against oxidant-mediated cytotoxicity via *S*-nitrosylation of GSH.

In our studies, SNAP, but not SNP, *S*-nitrosylated the active site thiols and protected them from DTT-insensitive higher oxidation: in other words, *S*-nitrosylation protected the thiols from NO-mediated oxidation. Figure 7 illustrates the relationship between thiol oxidation and *S*-nitrosylation. The *S*-nitrosylated thiols of enzyme can be rapidly restored by DTT or endogenous reductants such as GSH, although *S*-nitrosylation itself inhibits enzyme activity. DTT and GSH also reduce disulfide to thiol but not sulfenic acid. Although arsenite reduces sulfenic acid to thiol *in vitro*, such a reduction does not occur *in vivo*. Therefore, sulfenic acid is further oxidized to sulfinic or sulfonic acid in the presence of stronger oxidants such as N_2O_3 and NO_2, resulting in irreversible inactivation of the enzyme. Thus, the protective effect of NO on the thiol enzyme via *S*-nitrosylation from irreversible oxidation was shown. It will be interesting to explore the role of *S*-nitrosylation in physiological or pathological events mediated by NO.

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