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Mechanisms of Inhibition of Aldehyde Dehydrogenase by Nitroxyl, the Active Metabolite of the Alcohol Deterrent Agent Cyanamide

Eugene G. DeMaster,† Beth Redfern* and Herbert T. Nagasawa*‡*

*Medical Research Laboratories, Veterans Affairs Medical Center, Minneapolis, MN 55417; and
‡Department of Medicinal Chemistry, University of Minnesota, Minneapolis, MN 55455, U.S.A.

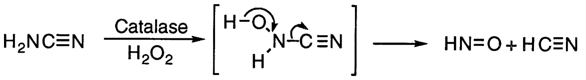
ABSTRACT. Nitroxyl, produced in the bioactivation of the alcohol deterrent agent cyanamide, is a potent inhibitor of aldehyde dehydrogenase (AlDH); however, the mechanism of inhibition of AlDH by nitroxyl has not been described previously. Nitroxyl is also generated from Angeli's salt ($Na_2N_2O_3$) at physiological pH, and, indeed, Angeli's salt inhibited yeast AlDH in a time- and concentration-dependent manner, with IC_{50} values under anaerobic conditions with and without NAD^+ of 1.3 and 1.8 μM, respectively. Benzaldehyde, a substrate for AlDH, competitively blocked the inhibition of this enzyme by nitroxyl in the presence of NAD^+, but not in its absence, in accord with the ordered mechanism of this reaction. The sulfhydryl reagents dithiothreitol (5 mM) and reduced glutathione (10 mM) completely blocked the inhibition of AlDH by Angeli's salt. These thiols were also able to partially restore activity to the nitroxyl-inhibited enzyme, the extent of reactivation being dependent on the pH at which the inactivation occurred. This pH dependency indicates the formation of two inhibited forms of the enzyme, with an irreversible form predominant at pH 7.5 and below, and a reversible form predominant at pH 8.5 and above. The reversible form of the inhibited enzyme is postulated to be an intra-subunit disulfide, while the irreversible form is postulated to be a sulfinamide. Both forms of the inhibited enzyme are derived via a common *N*-hydroxysulfenamide intermediate produced by the addition of nitroxyl to active site cysteine thiol(s). BIOCHEM PHARMACOL **55**;12:2007–2015, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. aldehyde dehydrogenase inhibition; Angeli's salt; cyanamide; dithiothreitol; glyceraldehyde-3-phosphate dehydrogenase inhibition; nitric oxide; nitroxyl

Cyanamide is used as an alcohol deterrent agent in Europe and Canada in the form of its citrated calcium salt (Dipsan®, Abstem®, and Temposil®) and in Japan as formulated aqueous solutions (Cyanamide Yoshitomi®). Following oral administration, cyanamide is absorbed rapidly from the GI tract and exerts its pharmacological action by inhibiting the low K_m mitochondrial AlDH§ (EC 1.2.1.3) and eliciting a carbimide–ethanol reaction [1] that mimics the better known disulfiram–ethanol reaction [2], thereby discouraging further ethanol consumption. The comparative clinical efficacy of cyanamide relative to disulfiram (Antabuse®) in alcohol-deterrent therapy has been reviewed [3, 4].

Cyanamide does not inhibit AlDH directly, but must be bioactivated *in vivo* to an active inhibitory species [5]. Its major urinary metabolite has been isolated from rats following [^{14}C]cyanamide administration, but was found to be inactive [6] when tested *in vitro*. We subsequently isolated this urinary metabolite, chemically characterized it as acetylcyanamide, and showed that acetylcyanamide is the major urinary conjugation product of cyanamide in the rat, rabbit, dog, and human [7]. Although 85–90% of orally administered cyanamide is metabolized and excreted as this inactive acetylcyanamide [7], cyanamide and its prodrug derivatives are the most potent *in vivo* inhibitors of AlDH known [8, 9].

We, and others, have also established that catalase is the enzyme that oxidatively converts cyanamide to a potent inhibitor of AlDH [10–13]. However, this catalase-catalyzed reaction represents a minor pathway in the overall disposition of cyanamide *in vivo*. Based on tracer studies with ^{15}N- and ^{13}C-labeled cyanamide, we hypothesized that the mechanism of bioactivation of cyanamide follows Equation 1, the putative inhibitor of AlDH being nitroxyl (HN═O). In the absence of a nucleophile, nitroxyl readily dimerizes to hyponitrous acid, which, in turn, dehydrates to N_2O and water (Equation 2). Detection of ^{15}N-labeled



(1)

$$2\ HN{=}O \longrightarrow \underset{\text{Hyponitrous Acid}}{[HON{=}NOH]} \longrightarrow N_2O + H_2O \qquad (2)$$

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† Corresponding author: Eugene G. DeMaster, Ph.D., Medical Research Laboratories (151), VA Medical Center, One Veterans Drive, Minneapolis, MN 55417. Tel. (612) 725-2000, Ext. 2854 or 2828; FAX (612) 725-2093.

§ *Abbreviations:* AlDH, aldehyde dehydrogenase; DEA/NO, diethylamine nonoate; DTT, dithiothreitol; GAP, glyceraldehyde-3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NO, nitric oxide; N_2O, nitrous oxide; and SULFI/NO, *N*-hydroxy-*N*-nitroso sulfamic acid.

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N_2O by GC/MS and ^{13}C-labeled HCN by Fourier-transformed-NMR spectroscopy constituted proof of their metabolic derivation from the corresponding isotopically labeled cyanamide [14].

Angeli's salt ($Na_2N_2O_3$, sodium trioxodinitrate) and Piloty's acid (benzenesulfohydroxamic acid) are chemical nitroxyl donors that liberate nitroxyl at physiologic and alkaline pH levels, respectively [15, 16], and are, therefore, convenient sources of this reactive metabolite of cyanamide. Indeed, Piloty's acid is a potent inhibitor of yeast AlDH even at physiologic pH with an IC_{50} of 48 μM [17]. In this study, we show that nitroxyl liberated from Angeli's salt also inhibits yeast AlDH and that the active site cysteine sulfhydryl group(s) is modified by nitroxyl. Our results also indicated that nitroxyl produces two inhibited forms of the enzyme, one form that can be reactivated by added thiols and a second form that is unreactive with these thiols. Molecular mechanisms for the inhibition of AlDH by nitroxyl are presented to reconcile the two forms of the inhibited enzyme.

MATERIALS AND METHODS

Materials

DTT, GSH, NAD^+, rabbit skeletal muscle GAPDH, cyanamide, *N*-acetyl-L-cysteine, and the diethyl acetal derivative of D,L-GAP (monobarium salt) were purchased from the Sigma Chemical Co. The barium salt of the GAP derivative was converted to the potassium salt of the free aldehyde as previously described [5]. Stock solutions of NO (approximately 2.0 mM) were prepared in deionized water from NO gas (Matheson Gas Products) as previously described [18]. **CAUTION:** *NO gas is highly toxic, and, therefore, the preparation of the 2.0 mM of stock solution of gaseous NO must be prepared in a fumehood*! DEA/NO was purchased from Cayman Chemical, and SULFI/NO was provided by Dr. Larry Keefer, NCI, NIH. Angeli's salt was prepared as described by Hunt *et al.* [19]. Stock solutions of DEA/NO, SULFI/NO, and Angeli's salt were prepared in deoxygenated 10 mM of KOH. Yeast AlDH (EC 1.2.1.5) was obtained from Boehringer Mannheim and dialyzed overnight against 20 mM of potassium phosphate buffer (pH 7.4) containing 0.2 M of KCl at 4° in a nitrogen atmosphere before use.

Inhibition Studies with AlDH

The effect of nitroxyl-based and other inhibitors on the activity of yeast AlDH was measured using a two-step assay system as previously described [20]. The primary reactions were carried out in sealed 0.74-mL reaction vessels under aerobic conditions, unless noted otherwise. The thiols, AlDH, and inhibitors were added through the septum of the reaction vessel by syringe. For anaerobic conditions, all solutions were deoxygenated with argon before use. The standard primary reaction mixtures containing 100 mM of potassium phosphate (pH 7.4), and 1.0 mM of NAD^+, where indicated, were preincubated for 5 min at 37° (deoxygenated using a stream of argon during the last 3 min of the preincubation period) followed by the addition of 0.08 U yeast AlDH and 0–10 μL of inhibitor solution, giving a total volume of 0.1 mL in sealed reaction vessels (Pierce). Following a 10-min incubation period, a 20-μL aliquot of the primary mixture was removed and added to a cuvette containing 0.5 mM of NAD^+, 1.0 mM of EDTA, 30% glycerol, and 100 mM of potassium phosphate buffer (pH 8.0) in a final volume of 1.0 mL. This secondary reaction was initiated by the addition of benzaldehyde (0.6 μmol) and carried out at 25°. The activity of yeast AlDH was determined spectrophotometrically by following the increase in concentration of NADH (340 nm) over time.

Inhibition Studies with GAPDH

The inhibition of GAPDH by Angeli's salt was studied using a tandem assay system as described above for AlDH. For these studies, the control primary reaction mixture contained 1.0 mM of EDTA, 0.5 mM of NAD^+, 0.1 U of GAPDH, 20 mM of sodium arsenate (pH 7.4), or, when indicated, 20 mM of sodium arsenate and 50 mM of Tris–Cl (pH 8.6) in a final volume of 0.1 mL. The secondary reaction mixture contained 0.5 mM of NAD^+, 1.0 mM of EDTA, 20 mM of sodium arsenate, 50 mM of Tris–Cl (pH 8.6), and 1.0 mM of GAP. Other details were as described for AlDH.

Production and Analysis of N_2O

The catalase-mediated generation of nitroxyl from cyanamide was carried out in reaction mixtures containing 50 mM potassium phosphate (pH 7.0), a continuous H_2O_2 generating system consisting of 10 mM of glucose and 100 μg of glucose oxidase, 40 mM of cyanamide, and 2 mg of bovine liver catalase in a total volume of 2.0 mL. The reactions were conducted at 37° in 20-mL glass vials equipped with septum seals. The samples were preincubated for 5 min at 37°, glucose oxidase was added followed by *N*-acetyl-L-cysteine, where indicated, and catalase 15 sec later, and the reaction vessels were sealed. After incubation, the samples were assayed immediately without the use of a reaction quenching reagent. N_2O, the end product of nitroxyl dimerization/dehydration, was quantified by headspace gas chromatography using thermal conductivity detection as previously described [14].

Ethanol Oxidation by Catalase

The experimental conditions and composition of the incubation mixtures were the same as described for the catalase-mediated oxidation of cyanamide described above except that 30 mM of ethanol was added in place of cyanamide. After incubation for 10 min at 37°, the reactions were quenched by the addition of 0.2 mL of 5.5 N perchloric acid. The acetaldehyde produced was quantified by headspace gas chromatography as described earlier [21].

Analysis of the Sulfhydryl Content of AlDH

The AlDH sulfhydryl content was measured spectrophotometrically at 412 nm using 0.5 μmol of Ellman's reagent added directly to the reaction mixture [22]. These results were compared against a standard curve constructed using increasing concentrations of GSH.

Data Presentation

Experimental values are given as means ± SEM of triplicate samples, unless indicated otherwise.

RESULTS

Inhibition of AlDH by Nitroxyl Derived from Angeli's Salt

The inhibition of AlDH by nitroxyl derived from Angeli's salt proceeded in a time-dependent manner (data not shown). This inhibition was enhanced slightly by NAD$^+$. The IC_{50} values for Angeli's salt under anaerobic conditions with and without NAD$^+$ in the reaction mixtures were 1.3 and 1.8 μM, respectively (Fig. 1A). The presence of oxygen (aerobic) had no effect on the extent of AlDH inhibition by Angeli's salt (Fig. 1B). Nitrite (1.0 mM), the other decomposition product of Angeli's salt, exhibited no inhibition toward AlDH, as expected.

In the absence of nucleophiles, nitroxyl dimerizes to hyponitrous acid, which subsequently decomposes to form N_2O and water (Equation 2). To test whether hyponitrous acid is inhibitory, the effect of SULFI/NO on AlDH activity was determined. SULFI/NO decomposes directly to hyponitrous acid, i.e. without the intermediacy of nitroxyl [23]. Using the same conditions as in Fig. 1, SULFI/NO (1.0 mM) reduced AlDH activity <20% compared with controls (results not shown).

Blockade of Nitroxyl Inhibition of AlDH by Benzaldehyde

According to the ordered mechanism of AlDH [24], NAD$^+$ binds to this enzyme before benzaldehyde, and therefore, benzaldehyde would be expected to block enzyme inhibition by nitroxyl in the presence, but not in the absence, of NAD$^+$. Indeed, benzaldehyde significantly blocked the inhibition of AlDH by Angeli's salt (10 μM) in the presence of NAD$^+$ (Fig. 2), whereas in the absence of NAD$^+$, no protection was observed even at a benzaldehyde concentration of 10 mM.

Blockade of Nitroxyl Inhibition of AlDH by Thiols

The ability of thiols to protect AlDH from inhibition by nitroxyl is based on the reactivity of sulfhydryl groups toward nitroxyl [23, 25, 26]. For example, nitroxyl derived from cyanamide [14], as determined by the amount of N_2O generated in the presence of *N*-acetyl-L-cysteine, was attenuated in a concentration-dependent manner (Fig. 3). However, because thiols may inhibit catalase activity [27], the effect of 10 μmol *N*-acetyl-L-cysteine on the catalase-catalyzed peroxidative oxidation of ethanol to acetaldehyde was assessed. The amount of acetaldehyde formed over 10 min with and without *N*-acetyl-L-cysteine (5.0 mM) was 2.02 ± 0.11 and 2.02 ± 0.15 μmol, respectively, thus indicating that catalase was not inhibited by *N*-acetyl-L-cysteine under these conditions.

The blockade of nitroxyl inhibition of AlDH by DTT or GSH was also evaluated using Angeli's salt. These reactions were carried out under anaerobic conditions to minimize air oxidation of the added thiols. DTT (5 mM) completely blocked inhibition of AlDH by nitroxyl (Fig. 4). Similarly, 10 mM of GSH with a sulfhydryl content equivalent to 5 mM of DTT also completely protected the enzyme from inhibition by nitroxyl, both in the presence and absence of NAD$^+$ (results not shown).

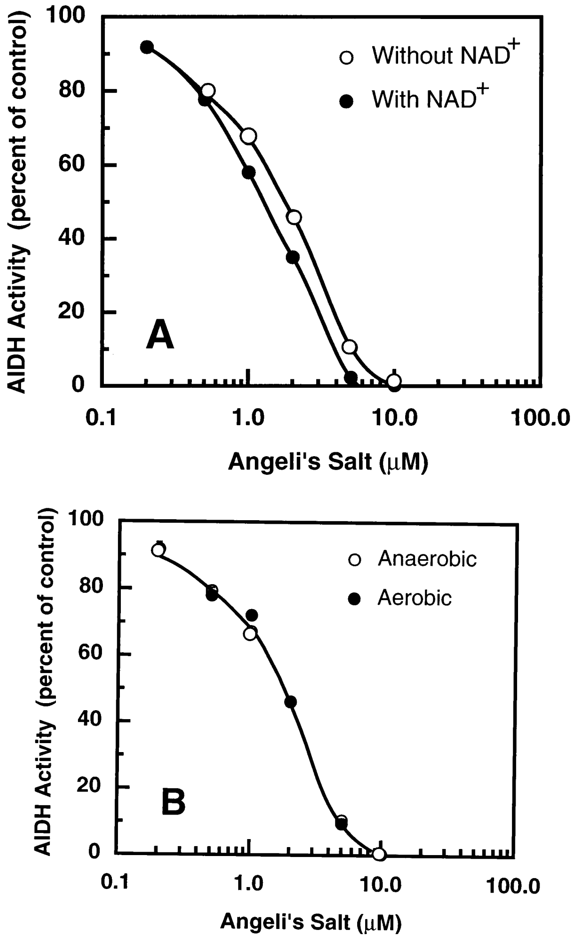


FIG. 1. Effect of NAD$^+$ (panel A) and oxygen (panel B) on the inhibition of AlDH by Angeli's salt. The concentration of NAD$^+$, where indicated, was 1.0 mM. Other experimental details are given under Materials and Methods. Control AlDH activities in panels A and B were 13.9 ± 0.4 and 13.8 ± 0.4 nmol NADH formed/min, respectively, in the secondary mixtures. Values are means ± SEM of triplicate samples.

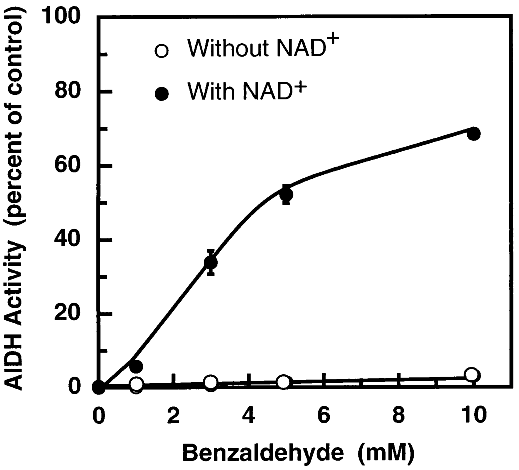


FIG. 2. Benzaldehyde blockade of the inhibition of AlDH by nitroxyl derived from Angeli's salt, with and without NAD^+. Benzaldehyde and NAD^+ (1.0 mM) were added to the primary incubation mixture before the addition of Angeli's salt. Control AlDH activity was 15.5 ± 0.1 nmol of NADH formed/min in the secondary reaction mixture. Values are means ± SEM of triplicate samples.

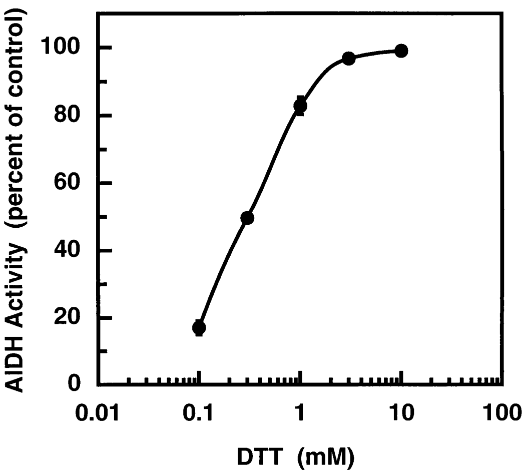


FIG. 4. DTT blockade of the inhibition of AlDH by nitroxyl derived from Angeli's salt. The primary mixture contained 1.0 mM of NAD^+, and the reactions were carried out under anaerobic conditions. DTT was added to the primary mixture before Angeli's salt. Other experimental details were as in Materials and Methods. Control AlDH activity was 16.6 ± 0.2 nmol NADH formed/min in the secondary reaction mixture. Values are means ± SEM of triplicate samples.

Reactivation of Nitroxyl-inhibited AlDH by Thiols

The reactivation of inhibited AlDH was assessed by adding DTT or GSH following nitroxyl inhibition of the enzyme. Twenty-five percent of the lost activity was restored to the inhibited enzyme by DTT treatment under the conditions used in Fig. 5A. Since DTT treatment also increased enzyme activity in the controls, approximately 7% of this restored activity is attributed to the reduction of a pre-existing, inactive disulfide form of the enzyme. In contrast, GSH treatment was less effective in restoring activity (only 4–5%) to the inhibited enzyme (Fig. 5B).

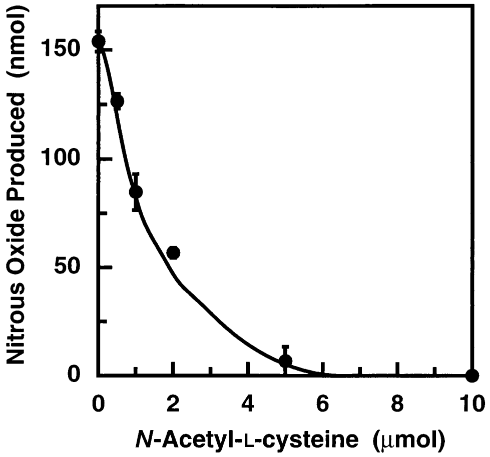


FIG. 3. Sequestration of nitroxyl derived from cyanamide by N-acetyl-L-cysteine as indicated by the attenuation of N_2O formation. The concentration-dependent sequestration of nitroxyl by N-acetyl-L-cysteine was carried out using a 10-min incubation period. The contents of complete reaction mixtures and other experimental details are given under Materials and Methods. Values are means ± SEM of triplicate samples.

The extent of reactivation of the nitroxyl (from Angeli's salt)-inhibited enzyme by DTT at physiologic pH was compared with the DTT reactivation of AlDH inhibited by other inhibitors of this enzyme, namely, nitroxyl derived from cyanamide, gaseous NO, NO from DEA/NO, H_2O_2, and acrolein (Table 1). When AlDH was inhibited by nitroxyl-based inhibitors (or by acrolein), less than 25% of the original enzyme activity was restored by DTT, whereas DTT restored more than 80% of the lost enzyme activity when AlDH was inactivated by NO-based inhibitors (or by H_2O_2). From these results, it is clear that different forms of inhibited AlDH can be produced. Moreover, some of these inhibited forms were reversible, i.e. activity could be restored by treatment with DTT, while others were irreversible, i.e. activity could not be restored by added DTT.

In further studies, it was found that the reactivation of the nitroxyl-inhibited enzyme by DTT was dependent on the pH at which the inhibition reaction was carried out (Fig. 6). When AlDH was inhibited by nitroxyl derived from Angeli's salt over a pH range of 6.0 to 9.0, the inhibited enzyme produced at pH 7.0 and below was unresponsive to reactivation by DTT, whereas more than 50% of the activity of the inhibited enzyme produced at pH 8.5 and above was restored by DTT. The rate of the decomposition of Angeli's salt to nitroxyl is constant over this pH range [15]. These results suggest that nitroxyl can produce two distinctly different inhibited forms of the enzyme: 1) an irreversibly inhibited form; and 2) an inhibited form that can be reactivated by DTT, the relative amount of each form produced being highly dependent on the pH at which the inactivation occurs.

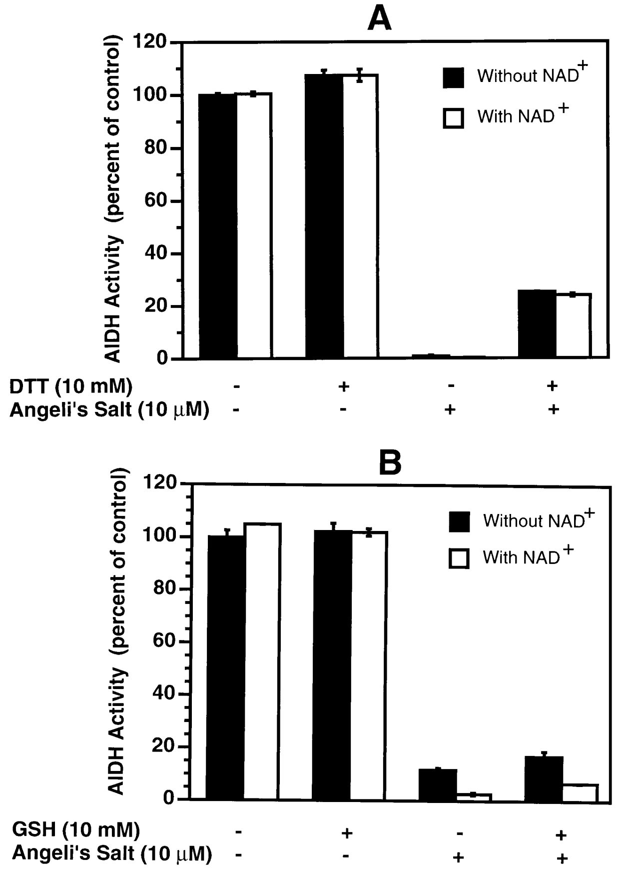


FIG. 5. Restoration of activity to nitroxyl-inhibited AlDH by DTT (panel A) and by GSH (panel B), with and without NAD$^+$. The experimental conditions were as in Fig. 1, except that the thiols were added to the primary mixture after 10 min of incubation. Aliquots of the primary mixture for AlDH activity measurements were taken 3 min after thiol addition. The reactions were carried out with 1.0 mM of NAD$^+$, where indicated, and under anaerobic conditions. Control AlDH activities with and without NAD$^+$ were 15.4 ± 0.1 and 15.3 ± 0.1 (panel A) and 14.3 ± 0.1 and 13.6 ± 0.4 (panel B) nmol NADH formed/min, respectively, in the secondary mixture. Values are means ± SEM of a minimum of triplicate samples.

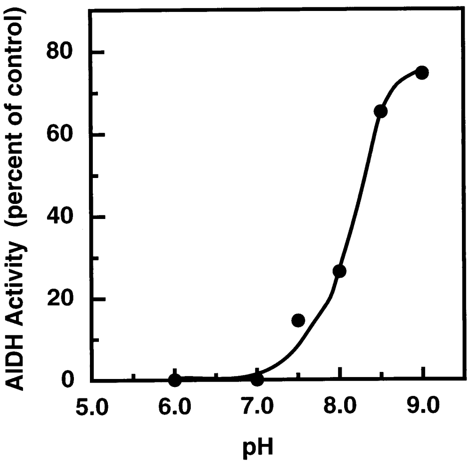


FIG. 6. Dependency of reactivation of nitroxyl-inhibited AlDH by DTT (10 mM) on the pH at which inactivation occurred. Control AlDH activities varied from 11.9 ± 0.1 (pH 9.0) to 15.3 ± 0.3 (pH 8.0) nmol of NADH formed/min in the secondary reaction mixture. Values are means ± SEM of four samples.

No Reactivation of Nitroxyl-inhibited GAPDH by DTT

There are many mechanistic similarities between AlDH and GAPDH [24]; however, GAPDH differs uniquely from AlDH in that it contains a single sulfhydryl group at its active center [28, 29] as opposed to AlDH enzymes that are characterized by the presence of proximal sulfhydryl groups at the active site [30, 31]. Although GAPDH does contain two reactive Cys sulfhydryl groups per monomer, namely, active site Cys-149 and Cys-153, these two sulfhydryl groups are not in close proximity in the stable, folded structure of the enzyme [32], but can form a disulfide in the denatured protein [33]. Selective alkylation of Cys-153 does not reduce enzyme activity markedly [32].

At physiologic pH, the IC_{50} for the inhibition of GAPDH by Angeli's salt was 0.80 μM (data not shown). In the

TABLE 1. Inhibition of yeast AlDH by DEA/NO, Angeli's salt, H_2O_2, acrolein, and cyanamide and reactivation by DTT*

Inhibitor	Inhibitor concentration (mM)	AlDH activity Before DTT addition (% of control)	AlDH activity After DTT addition (% of control)
None	0.0	100 ± 1	100 ± 2
DEA/NO†	0.15	<1	86 ± 2
Gaseous NO†	0.2	<1	87 ± 1
H_2O_2	4.0	23 ± 1	84 ± 3
Angeli's salt†	0.01	<1	24 ± 1
Cyanamide‡	1.0	<1	1 ± 0
Acrolein	1.0	<1	<1

*All primary incubations contained 1.0 mM of NAD$^+$ and were carried out under aerobic conditions, except where noted, and at 37° and pH 7.4. Gaseous NO was incubated with AlDH for 3 min, DEA/NO was incubated for 6 min, and the other inhibitors were incubated for 10 min before addition of DTT (10 mM). AlDH activity was determined at 10 or 3 min after the addition of DTT. Control AlDH activities, i.e. before and after DTT addition, were 24.7 ± 0.2 and 30.5 ± 0.2 nmol of NADH formed/min, respectively. Values are means ± SEM of triplicate samples.

†Reactions were carried out under anaerobic conditions.

‡Incubation mixture also included 10 mM of glucose, 1.3 μg of glucose oxidase, and 2.5 μg of catalase.

presence of 10 μM of Angeli's salt under anaerobic conditions, GAPDH was inhibited completely after 10 min at either pH 7.4 or 8.6. The addition of 10 mM of DTT did not reactivate the nitroxyl-inhibited enzyme inactivated at either pH (data not shown).

Sulfhydryl Content of Nitroxyl-inhibited AlDH

The tetrameric yeast enzyme has eight readily titratable (Ellman's reagent) sulfhydryl groups, two per subunit [34]. Previously, we showed that the inhibition of AlDH by NO was characterized by the formation of two intra-subunit disulfide bonds and that this inactive enzyme still retained four of its eight sulfhydryl groups in the reduced form [23]. The latter is in agreement with "half-of-the-site" reactivity for tetrameric yeast AlDH [34].

In a parallel experiment, yeast AlDH was exposed to 10 μM of Angeli's salt in the presence of 1.0 mM of NAD^+ under the same conditions as given in Fig. 1A, except that the reaction mixtures were scaled up 10-fold. Following a 10-min incubation period, the activity of the enzyme was found to be inhibited greater than 99% compared with similarly treated controls without Angeli's salt. The sulfhydryl content of the nitroxyl-inhibited enzyme was 45.3 ± 3.3% (N = 4) vs 100.0 ± 2.1% for the controls (N = 4). Thus, like NO, nitroxyl from Angeli's salt modified approximately four of the eight titratable sulfhydryl groups of yeast AlDH. Possible molecular modifications of the Cys thiols of AlDH by nitroxyl are presented in the Discussion that follows.

DISCUSSION

Our results show that nitroxyl generated *in situ* from Angeli's salt inhibited AlDH in a time- and concentration-dependent manner with an IC_{50} of less than 2 μM for Angeli's salt. The modest enhancement of inhibition by NAD^+ (Fig. 1) and the blockade of inhibition by the substrate benzaldehyde (Fig. 2) are consistent with an interaction between nitroxyl and the active site cysteine as well as the ordered mechanism of this reaction [2]. While nitroxyl can be oxidized to NO by molecular oxygen [35, 36], the presence of oxygen did not affect the IC_{50} of this inhibition (Fig. 1).

The inhibitor constant for nitroxyl itself cannot be determined because the concentration of nitroxyl in solution is unknown. The concentration of nitroxyl is dependent not only on its rate of production from Angeli's salt, which occurs according to a first order reaction [37], but also on the unknown rate of dimerization of nitroxyl to hyponitrous acid (Equation 2). Moreover, the concentration of nitroxyl would not be expected to remain constant over the total period of incubation. As an alternative, the IC_{50} values of the pro forms of nitroxyl, i.e. the nitroxyl donors themselves, are used to compare their relative inhibitory potencies. For example, the IC_{50} values for the inhibition of yeast AlDH by Angeli's salt and Piloty's acid [17] under physiological conditions were found to be 1.3 and 48 μM, respectively.

Yeast and most mammalian AlDH isozymes contain juxtaposed sulfhydryl groups at their active centers [23, 30, 31, 38]. The oxidation of these thiols, e.g. by NO, in the yeast enzyme results in the formation of intra-subunit disulfide bonds and the observed loss of enzyme activity [23]. These thiols also appear to be the targets for the modification of AlDH by nitroxyl. The reactivity of nitroxyl with thiols was confirmed by the ability of *N*-acetyl-L-cysteine to sequester nitroxyl, thereby decreasing N_2O formation from cyanamide oxidation (Fig. 3), and by the blockade of nitroxyl inhibition of AlDH by added DTT (Fig. 4) or GSH, which act as competitive "traps" for nitroxyl. Moreover, the presence of the aldehyde substrate benzaldehyde, which forms a thiohemiacetal with the active site Cys-302, also blocked inhibition of AlDH by nitroxyl (Fig. 2).

Inhibition of AlDH by nitroxyl produced two distinct inhibited forms of the enzyme, namely, a reversibly inhibited form that could be reactivated by DTT and an irreversibly inhibited form unresponsive to DTT treatment. The irreversible form was produced exclusively at pH 7.0 and below, whereas the primary form produced at pH 8.5 and above was reversible (Fig. 6). The latter appears to be identical to the inhibited enzyme produced by NO [23]. Indeed, NO, nitroxyl and H_2O_2 are known to oxidize thiols to disulfides [23, 24, 26, 39–43], and each of these agents also inhibits AlDH to produce an inhibited form of the enzyme that can be reactivated by DTT (Table 1 and Fig. 6). Moreover, GAPDH, which contains only a single sulfhydryl group at its active center [28, 29] and catalyzes reactions mechanistically similar to that carried out by AlDH, was irreversibly inhibited by nitroxyl at both pH 7.4 and 8.6. Based on these considerations, we conclude that the formation of the reversible form of the inhibited enzyme involves the net oxidation of proximal active site cysteine thiols, yielding intra-subunit disulfide bonds as observed for the inhibition of AlDH by NO [23].

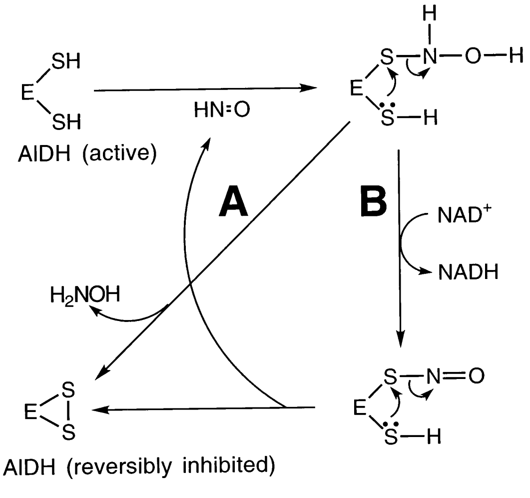


FIG. 7. Proposed mechanisms for the *reversible* inactivation of AlDH by nitroxyl-based inhibitors of AlDH.

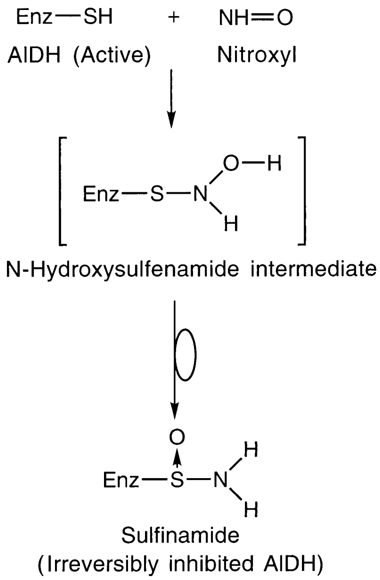


FIG. 8. Proposed mechanism for the *irreversible* inactivation of AlDH by nitroxyl-based inhibitors of AlDH.

Two possible mechanisms for the oxidation of the proximal active site thiols to a disulfide are given in Fig. 7. The initial reaction of nitroxyl with a sulfhydryl group produces an *N*-hydroxysulfenamide intermediate. In pathway A, the intramolecular expulsion of hydroxylamine by a neighboring sulfhydryl nucleophile gives the disulfide form of the inhibited enzyme. This pathway is typified by the model reaction of DTT with nitroxyl, which yields hydroxylamine and the cyclic disulfide of DTT [23, 40]. In pathway B, the *N*-hydroxysulfenamide intermediate is oxidized by NAD^+ to a new *S*-nitroso intermediate, which undergoes intrasubunit disulfide formation with elimination of nitroxyl.

Our mechanism for the irreversible inhibition of AlDH by nitroxyl (Fig. 8) is based in part on the chemistry of the reaction of aromatic nitroso substances with thiols [41] and in part on our own studies on the interaction of model thiol compounds with nitroxyl [44]. Nitroxyl reacts with an active site sulfhydryl group, i.e. Cys-302 [45], producing an *N*-hydroxysulfenamide intermediate that undergoes an intramolecular rearrangement at lower pH levels to yield a sulfinamide. The resulting thiol modified sulfinamide would be expected to be resistant to reduction by DTT, thus accounting for the irreversibility of the inhibition.

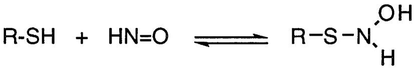
Analysis of the enzyme thiols remaining after irreversible inhibition of AlDH by nitroxyl indicated that both thiols of the active subunits were modified. Using Ellman's reagent, yeast AlDH has been shown to contain eight readily titratable sulfhydryl groups—presumably two per subunit [46]. As observed for the inhibition of this enzyme by NO [23], complete inhibition of AlDH by nitroxyl was accompanied by a loss of approximately half of the titratable sulfhydryl groups. By analogy with our earlier studies with AlDH and NO, the sulfhydryl groups of the two active subunits of the tetrameric enzyme were selectively modified by nitroxyl. These results are consistent with "half-of-the-site" reactivity for the tetrameric yeast and mammalian isozymes of AlDH [34, 47], i.e. only two of the four subunits of AlDH are catalytically active at physiologic pH.

The chemical evidence for the formation of a disulfide in certain AlDH isozymes is convincing. Our work on the inhibition of yeast AlDH by NO [23], studies on the inhibition of sheep [38] and human [31] class 1 AlDH by disulfiram, and the investigations described here with nitroxyl (Fig. 7) all indicate that disulfide formation accompanies enzyme inhibition with these inhibitors. What remains unclear is the location of the second Cys residue in the primary structure of these isozymes of AlDH that participate in this disulfide bond formation.

The tertiary structures of a class 2 and a class 3 AlDH isozyme have been reported recently [48, 49]. Attempts were made to identify the putative proximal Cys residue that forms a disulfide with the active site Cys, i.e. residue 302 in bovine class 2 AlDH and residue 243 in rat class 3 AlDH. This active site Cys residue is the only Cys in the primary structure that is conserved across a broad phylogenetic distribution of AlDH isozymes [50]. It was not possible to visualize the second Cys residue without invoking significant structural modification prior to disulfide bond formation. Moreover, because thiols can restore enzyme activity, such a structural modification would need to be reversible. Alternatively, all of the AlDH isozymes reported to date that form a disulfide following treatment with an inhibitor contain a Cys residue adjacent to the active site Cys (nucleophile) in their primary structures. However, there is no precedent for the formation of a stable disulfide between two adjacent Cys residues, and there are some steric concerns regarding the formation of an eight-membered ring disulfide between two adjacent Cys residues.

While nitroxyl is clearly the metabolite responsible for inhibition of AlDH by cyanamide *in vivo*, less clear is whether nitroxyl itself is the actual inhibitory species *in vivo* as described for the inhibition of AlDH *in vitro* (Figs. 7 and 8), or whether the redox partners of nitroxyl [51], namely, NO and the nitrosonium ion (NO^+) as an *S*-nitrosothiol, participate in this inhibition. The interconversion of nitroxyl and NO is catalyzed by superoxide dismutase [35, 36], while *S*-nitrosothiols, such as *S*-nitrosoglutathione, are formed from a thiol and NO in the presence of oxygen via a nitrosylation reaction [52]. *S*-Nitrosothiols produce NO by homolytic cleavage [53, 54] and nitroxyl through reduction by another thiol [23, 40]. Because all three of these reactive oxides of nitrogen inhibit AlDH *in vitro* [23, 55], it is not possible, at present, to assign the inhibition of AlDH *in vivo* to a single reactive oxide of nitrogen.

These interconversions between nitroxyl and its redox partners may also explain, in part, how a reactive substance like nitroxyl, which is produced in peroxisomes from cyanamide by catalase, can inhibit AlDH2 located in the mitochondria. For example, NO produced from L-arginine in endothelial cells activates guanylate cyclase in platelets and smooth muscle cells [56]. If NO formed in one cell can exert an effect in another cell, surely NO can affect the activity of an enzyme located in a different compartment within the same cell. In addition, nitroxyl may diffuse through membranes either as free nitroxyl or be transported as an *N*-hydroxysulfenamide of GSH or cysteine (Equation 3). Finally, *S*- nitrosothiols are considered relatively stable carrier forms of the redox partners of nitroxyl [51]. The transport/diffusion and metabolic fate of nitroxyl, NO, and the nitrosonium ion (as *S*-nitrosothiols) in complex biological matrices are areas currently under intense investigation.



$$\mathrm{R\text{-}SH + HN{=}O \rightleftharpoons R{-}S{-}N(OH)H}$$

(3)

In summary, nitroxyl produces two forms of inhibited AlDH, one form that can be reactivated by DTT and a second that is resistant to reactivation by DTT. The mechanism for the reversible inhibition of the enzyme by nitroxyl involves the oxidation of the proximal active site cysteine thiols to give an intra-subunit disulfide identical to that observed following inhibition by NO [23]. In contrast, the mechanism for the irreversible inhibition by nitroxyl is suggested to involve an intramolecular rearrangement of the initially formed *N*-hydroxysulfenamide intermediate to a sulfinamide, thereby irreversibly modifying the active site sulfhydryl groups of the enzyme.

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References

1. Brien JF, Peachey JE and Loomis CW, Calcium carbimide–ethanol interaction. *Clin Pharmacol Ther* **27:** 426–433, 1980.
2. Kitson TM, The disulfiram–ethanol reaction. *J Stud Alcohol* **38:** 96–113, 1977.
3. Sellers EM, Naranjo CA and Peachey JE, Drugs to decrease alcohol consumption. *N Engl J Med* **305:** 1255–1262, 1981.
4. Peachey JE, A review of the clinical use of disulfiram and calcium carbimide in alcoholism treatment. *J Clin Psychopharmacol* **1:** 368–375, 1981.
5. DeMaster EG, Kaplan E, Shirota FN and Nagasawa HT, Metabolic activation of cyanamide by liver mitochondria, a requirement for the inhibition of aldehyde dehydrogenase enzymes. *Biochem Biophys Res Commun* **107:** 1333–1339, 1982.
6. Deitrich RA, Troxell PA and Worth WS, Inhibition of aldehyde dehydrogenase in brain and liver by cyanamide. *Biochem Pharmacol* **25:** 2733–2737, 1976.
7. Shirota FN, Nagasawa HT, Kwon CH and DeMaster EG, *N*-Acetylcyanamide, the major urinary cyanamide metabolite in rat, rabbit, dog, and man. *Drug Metab Dispos* **12:** 337–344, 1984.
8. Nagasawa HT, Kwon CH, DeMaster EG and Shirota FN, Prodrugs of cyanamide as (long-acting) alcohol deterrent agents. *Biochem Pharmacol* **35:** 129–132, 1986.
9. Kwon CH, Nagasawa HT, DeMaster EG and Shirota FN, Acyl, *N*-protected α-aminoacyl, and peptidyl derivatives as prodrug forms of the alcohol deterrent agent cyanamide. *J Med Chem* **29:** 1922–1929, 1986.
10. DeMaster EG, Shirota FN and Nagasawa HT, The metabolic activation of cyanamide to an inhibitor of aldehyde dehydrogenase is catalyzed by catalase. *Biochem Biophys Res Commun* **122:** 358–365, 1984.
11. DeMaster EG, Shirota FN and Nagasawa HT, Catalase mediated conversion of cyanamide to an inhibitor of aldehyde dehydrogenase. *Alcohol* **2:** 117–121, 1985.
12. Svanas GW and Weiner H, Enzymatic requirement for cyanamide inactivation of rat liver aldehyde dehydrogenase. *Biochem Pharmacol* **34:** 1197–1204, 1985.
13. Shirota FN, DeMaster EG and Nagasawa HT, Cyanide is a product of the catalase-mediated oxidation of the alcohol deterrent agent, cyanamide. *Toxicol Lett* **37:** 7–12, 1987.
14. Nagasawa HT, DeMaster EG, Redfern B, Shirota FN and Goon DJW, Evidence for nitroxyl in the catalase-mediated bioactivation of the alcohol deterrent agent cyanamide. *J Med Chem* **33:** 3120–3122, 1990.
15. Hughes MN and Wimbledon PE, The chemistry of trioxodinitrates. Part I. Decomposition of sodium trioxodinitrate (Angeli's salt) in aqueous solution. *J Chem Soc Dalton* 703–707, 1976.
16. Bonner FT and Ko Y, Kinetic, isotopic, and ^{15}N NMR study of *N*-hydroxybenzenesulfonamide decomposition: An HNO source reaction. *Inorgan Chem* **31:** 2514–2519, 1992.
17. Nagasawa HT, Kawle SP, Elberling JA, DeMaster EG and Fukuto JM, Prodrugs of nitroxyl as potential aldehyde dehydrogenase inhibitors vis-a-vis vascular smooth muscle relaxants. *J Med Chem* **38:** 1865–1871, 1995.
18. Archer SL, Shultz PJ, Warren JB, Hampl V and DeMaster EG, Preparation of standards and measurement of nitric oxide, nitroxyl and related oxidation products. *Methods Companion Methods Enzymol* **7:** 21–34, 1995.
19. Hunt HR Jr, Cox JR Jr and Ray JD, The heat of formation of crystalline sodium α-oxyhyponitrite. The structure of aqueous α-oxyhyponitrite ion. *Inorgan Chem* **1:** 938–941, 1962.
20. Nagasawa HT, Yost Y, Elberling JA, Shirota FN and DeMaster EG, Nitroxyl analogs as inhibitors of aldehyde dehydrogenase. C-Nitroso compounds. *Biochem Pharmacol* **45:** 2129–2134, 1993.
21. DeMaster EG, Dahlseid T and Redfern B, Comparative oxidation of 2-propyn-1-ol with other low molecular weight unsaturated and saturated primary alcohols by bovine liver catalase *in vitro*. *Chem Res Toxicol* **7:** 414–419, 1994.
22. Habeeb AFSA, Reaction of protein sulfhydryl groups with Ellman's reagent. *Methods Enzymol* **25:** 457–464, 1972.
23. DeMaster EG, Redfern B, Quast BJ, Dahlseid T and Nagasawa HT, Mechanism for the inhibition of aldehyde dehydrogenase by nitric oxide. *Alcohol* **14:** 181–189, 1997.
24. Dickinson FM, Aldehyde dehydrogenase: Kinetics and mechanism. *Biochem Soc Trans* **17:** 299–300, 1989.
25. Doyle MP, Mahapatro SN, Broene RD and Guy JK, Oxidation and reduction of hemoproteins by trioxodinitrate(II). The role of nitroxyl hydride and nitrite. *J Am Chem Soc* **110:** 593–599, 1988.
26. Turk T and Hollocher TC, Oxidation of dithiothreitol during turnover of nitric oxide reductase: Evidence for generation of nitroxyl with the enzyme from *Paracoccus denitrificans*. *Biochem Biophys Res Commun* **183:** 983–988, 1992.
27. Sun Y and Oberley LW, The inhibition of catalase by glutathione. *Free Radic Biol Med* **7:** 595–602, 1989.
28. Benitez LV and Allison WS, The inactivation of the acyl phosphatase activity catalyzed by the sulfenic acid form of glyceraldehyde-3-phosphate dehydrogenase by dimedone and olefins. *J Biol Chem* **249:** 6234–6243, 1974.
29. Padgett CM and Whorton AR, *S*-Nitrosoglutathione reversibly inhibits GAPDH by *S*-nitrosylation. *Am J Physiol* **269**(3 Pt 1)**:** C739–C749, 1995.
30. Jakoby WB, Aldehyde oxidation. II. Evidence for closely juxtaposed sulfhydryl groups on dehydrogenases. *J Biol Chem* **232:** 89–97, 1958.

31. Vallari RC and Pietruszko R, Human aldehyde dehydrogenase: Mechanism of inhibition of disulfiram. *Science* **216:** 637–639, 1982.
32. Moore J Jr and Fenselau A, Selective cysteine modification in glyceraldehyde-3-phosphate dehydrogenase. *Biochemistry* **11:** 3762–3770, 1972.
33. Wassarman PM and Major JP, The reactivity of the sulfhydryl groups of lobster muscle glyceraldehyde 3-phosphate dehydrogenase. *Biochemistry* **8:** 1076–1082, 1968.
34. Bradbury SL and Jakoby WB, Ligand interactions with yeast aldehyde dehydrogenase. *J Biol Chem* **246:** 6929–6932, 1971.
35. Murphy ME and Sies H, Reversible conversion of nitroxyl anion to nitric oxide by superoxide dismutase. *Proc Natl Acad Sci USA* **88:** 10860–10864, 1991.
36. Fukuto JM, Hobbs AJ and Ignarro LJ, Conversion of nitroxyl to nitric oxide in biological systems: The role of physiological oxidants and relevance to the biological activity of HNO. *Biochem Biophys Res Commun* **196:** 707–713, 1993.
37. Maragos CM, Morley D, Wink DA, Dunams TM, Saavedra JE, Hoffman A, Bove AA, Isaac L, Hrabie JA and Keefer LK, Complexes of $^{\bullet}$NO with nucleophiles as agents for the controlled biological release of nitric oxide. Vasorelaxant effects. *J Med Chem* **34:** 3242–3247, 1991.
38. Kitson TM, Mechanism of inactivation of sheep liver aldehyde dehydrogenase by disulfiram. *Biochem J* **213:** 551–554, 1983.
39. Pryor WA, Church DF, Govindan CK and Crack G, Oxidation of thiols by nitric oxide and nitrogen dioxide: Synthetic utility and toxicological implications. *J Org Chem* **47:** 156–159, 1982.
40. Arnelle DR and Stamler JS, NO^+, $NO^{\bullet}$, NO^- donation by *S*-nitrosothiols: Implications for regulation of physiological functions by *S*-nitrosylation and acceleration of disulfide formation. *Arch Biochem Biophys* **318:** 279–285, 1995.
41. Ellis MK, Hill S and Foster PMD, Reactions of nitrosobenzenes with biological thiols: Identification and reactivity of glutathion-*S*-yl conjugates. *Chem Biol Interact* **82:** 151–163, 1992.
42. Radi R, Beckman JS, Bush KM and Freeman BA, Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J Biol Chem* **266:** 4244–4250, 1991.
43. Barton JP, Packer JE and Sims RJ, Kinetics of the reaction of hydrogen peroxide with cysteine and cysteamine. *J Chem Soc Perkin Trans* **II:** 1547–1549, 1973.
44. Shoeman DW, Nagasawa HT and DeMaster EG, Trapping of nitroxyl (HNO) generated *in vitro* by sulfhydryl reagents. *FASEB J* **10:** A177, 1996.
45. Saigal D, Cunningham SJ, Farres J and Weiner H, Molecular cloning of the mitochondrial aldehyde dehydrogenase gene of *Saccharomyces cerevisiae* by genetic complementation. *J Bacteriol* **173:** 3199–3208, 1991.
46. Steinman CR and Jakoby WB, Yeast aldehyde dehydrogenase. II. Properties of the homogeneous enzyme preparations. *J Biol Chem* **243:** 730–734, 1968.
47. Takahashi K, Weiner H and Filmer DL, Effects of pH on horse liver aldehyde dehydrogenase: Alterations in metal ion activation, number of functioning active sites, and hydrolysis of the acyl intermediate. *Biochemistry* **20:** 6225–6235, 1981.
48. Steinmetz CG, Xie P, Weiner H and Hurley TD, Structure of mitochondrial aldehyde dehydrogenase: The genetic component of ethanol aversion. *Structure* **5:** 701–711, 1997.
49. Liu ZJ, Sun YJ, Rose J, Chung YJ, Hsiao CD, Chang WR, Kuo I, Perozich J, Lindahl R, Hempel J and Wang BC, The first structure of an aldehyde dehydrogenase reveals novel interactions between NAD and the Rossmann fold. *Nature Struct Biol* **4:** 317–326, 1997.
50. Hempel J, Nicholas H and Lindahl R, Aldehyde dehydrogenases: Widespread structural and functional diversity within a shared framework. *Protein Sci* **2:** 1890–1900, 1993.
51. Stamler JS, Singel DJ and Loscalzo J, Biochemistry of nitric oxide and its redox-activated forms. *Science* **258:** 1898–1902, 1992.
52. DeMaster EG, Quast BJ and Mitchell RA, Inhibition of *S*-nitrosation of reduced glutathione in aerobic solutions of nitric oxide by phosphate and other inorganic anions. *Biochem Pharmacol* **53:** 581–585, 1997.
53. Williams DLH, *S*-Nitrosothiols and role of metal ions in decomposition to nitric oxide. *Methods Enzymol* **268:** 299–307, 1996.
54. Singh RJ, Hogg N, Joseph J and Kalyanaraman B, Mechanism of nitric oxide release from *S*-nitrosothiols. *J Biol Chem* **271:** 18596–18603, 1996.
55. Shirota FN, DeMaster EG, Goon DJW and Nagasawa HT, Nitroxyl (HNO) formation *in vitro* from isoamylnitrite as well as from *S*-nitrosoglutathione. *Pharmacologist* **14:** 174, 1992.
56. Ignarro LJ, Signal transduction mechanisms involving nitric oxide. *Biochem Pharmacol* **41:** 485–490, 1991.