Biochemical Pharmacology, Vol. 55, pp. 2007–2015, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00080-X

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.

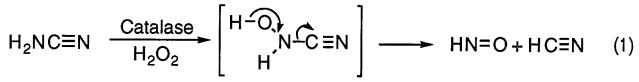
ABSTRACT. Nitroxyl, produced in the bioactivation of the alcohol deterrent agent cyanamide, is a potent inhibitor of aldehyde dehydrogenase (AlDH); however, the mechanism of inhibition of AlDH by nitroxyl has not been described previously. Nitroxyl is also generated from Angeli's salt ($Na_2N_2O_3$) 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

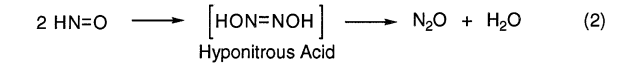
Cyanamide is used as an alcohol deterrent agent in Europe and Canada in the form of its citrated calcium salt (Dipsan®, Abstem®, and Temposil®) and in Japan as formulated aqueous solutions (Cyanamide Yoshitomi®). Following oral administration, cyanamide is absorbed rapidly from the GI tract and exerts 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



† 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.

Received 5 August 1997; accepted 27 January 1998.

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