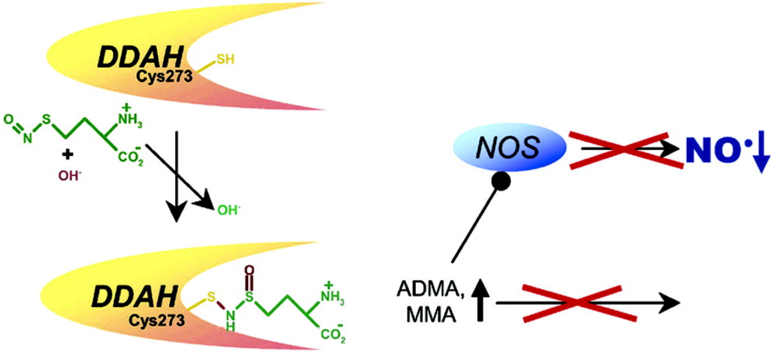
Communication

Searching for DDAH Inhibitors: *S*-Nitroso-l-homocysteine Is a Chemical Lead

Markus Knipp, Oliver Braun, and Milan Vak

J. Am. Chem. Soc., **2005**, 127 (8), 2372-2373• DOI: 10.1021/ja0430200 • Publication Date (Web): 03 February 2005



Journal of the American Chemical Society is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

J|A|C|S
COMMUNICATIONS

Searching for DDAH Inhibitors: *S*-Nitroso-L-homocysteine Is a Chemical Lead

Markus Knipp,* Oliver Braun, and Milan Vašák
Institute of Biochemistry, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

Received November 19, 2004; E-mail: knippy@bioc.unizh.ch

NO is a molecule of remarkable importance and a danger for biological systems at the same time.[1] Thus, there is substantial interest to control the activity of nitric oxide synthase (NOS) isoenzymes. For several diseases, such as septic shock, migraine, inflammation, and neurodegenerative disorders, NO production should be reduced. Therefore, inhibitors of NOS have been sought.[2] However, despite intense research no clinically useful NOS inhibitor is currently available. Therefore, NO regulation through increased concentrations of the endogenous NOS inhibitors N^{ω}-methyl-L-arginine (MMA) and N^{ω},N^{ω}-dimethyl-L-arginine (ADMA) is of emerging interest.[3] Moreover, by this approach detrimental complete inhibition of NOS may be avoided.

The enzyme responsible for the catabolism of MMA and ADMA is the Cys-hydrolase N^{ω},N^{ω}-dimethyl-L-arginine dimethylaminohydrolase (DDAH), which yields L-citrulline and $CH_3NH_3^+$ or $(CH_3)_2NH_2^+$, respectively. At present, there is only one noncovalent DDAH inhibitor of a very low affinity available.[4] Both known mammalian DDAH isoenzymes are targets for *S*-nitrosylation by free NO at their active-site Cys.[5] An important source of NO equivalents in the cytosol is endogenous *S*-nitrosothiols, mainly those of glutathione, L-cysteine, and L-homocysteine (HcyNO). While investigating their ability to interact with DDAH-1, we found a novel type of Cys modification by HcyNO that provides a basis for the rational design of new DDAH inhibitors.

When bovine DDAH-1 was incubated with HcyNO,[6] it appeared that HcyNO irreversibly inhibited DDAH-1 in a competitive manner and with a much higher specificity than free NO radicals (to be published elsewhere). Further analysis by electrospray ionization quadrupole time-of-flight (ESI Q-TOF) MS revealed a covalent product with a mass increase for DDAH-1 of 164.0 Da (Table 1). This mass difference corresponds to one HcyNO molecule.[7] However, because a covalent product was formed, HcyNO had to undergo conversion. Moreover, it was found that the modification was completely lost when the modified protein was unfolded in 6 M guanidinium·Cl or 8 M urea prior to MS analysis. Consequently, the product formed is stabilized by the protein structure at physiological pH.

Table 1. Masses of the Reaction Product of DDAH-1 with HcyNO[a]

reagents used[b]	mass (Da)	mass difference (Da)[c]
–	31199.1 ± 0.5[d]	–
500 μM HcyNO in H_2O	31363.1 ± 0.5[e]	164.0
500 μM $Hcy^{15}NO$ in H_2O	31364.1 ± 0.2[f]	165.0
500 μM $HcyN^{18}O$ in H_2O	31363.3 ± 0.3[f]	164.2
500 μM HcyNO in $H_2{}^{18}O$	31365.1 ± 0.3[f]	166.0

[a] Reactions were performed in 50 mM Hepes/NaOH (pH 7.4), 150 mM KCl, 5 mM EDTA for 30 min at 37 °C. [b] Atoms without mass numbers indicated were used at natural abundance. [c] Difference between the mass of native DDAH-1 and modified DDAH-1. [d] Mean value of seven experiments of native DDAH-1. [e] Mean value of nine experiments. [f] Mean value of three experiments.

To characterize the species formed, a series of ESI Q-TOF MS experiments using isotopically labeled HcyNO and H_2O was conducted (Table 1). Because the expected mass shifts were small compared to the total mass of DDAH-1, all experiments were performed in triplicate. In parallel to the isotopically labeled samples, unlabeled controls were always measured. As shown in Table 1, a mass shift could only be observed when $Hcy^{15}NO$ was used, but not $HcyN^{18}O$. Additional experiments in $H_2{}^{18}O$ showed that water is the source of the O atom. To address the question which species was formed, the structural similarity of the amino acid HcyNO to the substrate molecules should be considered first (Figure S1). In the X-ray structure of *Pseudomonas aeruginosa* DDAH the amino acids MMA, ADMA, and L-citrulline are anchored through their α-CO_2^- and α-NH_3^+ groups inside of the substrate channel of the enzyme, thus facilitating the correct side-chain orientation toward the active-site residues.[8] By analogy, a molecular model of DDAH in complex with HcyNO was built (Figure 1). In this model, Cys249:S was located 2.1 Å from HcyNO:N^{ϵ}, thus favoring a nucleophilic attack. Similarly to the DDAH·ADMA complex,[8] Asp66:O^{δ} may also form a hydrogen bridge with HcyNO:N^{ϵ}, thus increasing its electrophilicity. In contrast to ADMA, HcyNO may not be affected by His162.

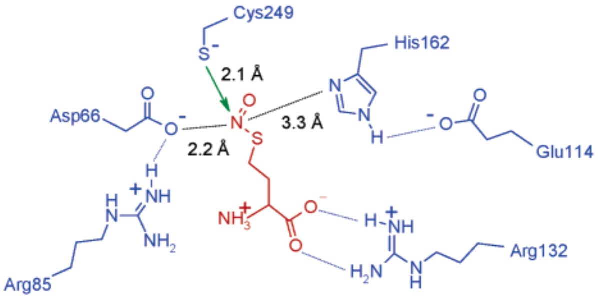
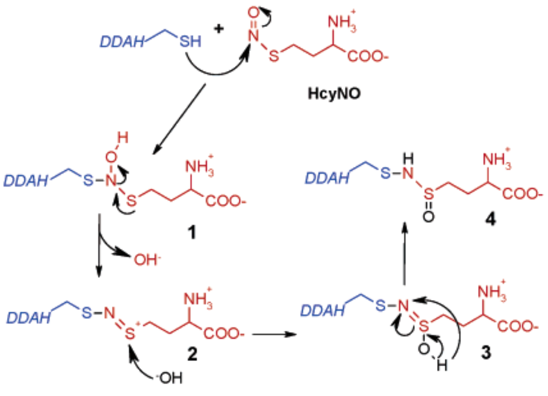


Figure 1. Active-site of the molecular model of DDAH (blue) with HcyNO (red) inserted. The model was obtained through computational energy minimization of *P. aeruginosa* DDAH(C249S) (PDB code 1h70) upon remutation of Ser249 into Cys (for details see Supporting Information).

A S_N2 attack of Cys249:S on HcyNO:N^{ϵ} may form the *N*-hydroxysulfinimide **1** (Scheme 1) that has been widely invoked

Scheme 1. Proposed Reaction between the Active-Site Cys273:S of DDAH-1 (blue) and HcyNO (red)



10.1021/ja0430200 CCC: $30.25 © 2005 American Chemical Society

to account for some *S*-nitrosothiol chemistry.[9] Because of its instability, **1** would decompose to form the sulfiliminosulfonium ion **2**. This mechanism is supported by the absence of a mass shift when $HcyN^{18}O$ was used instead of $HcyN^{16}O$ (Table 1). Although not isolated from the reaction of thiols with *S*-nitrosothiols,[9] sulfiliminosulfonium ions have been characterized upon the reaction of the isoelectronic *N*-chlorosulfimides with sulfides.[10] The O atom inserted from H_2O may be best explained by the attack of a water on the formally positively charged S atom of **2**. In the enzyme pocket, activated water molecules are present.[11] Thus, the reaction likely proceeds through the intermediate **3** to yield the *N*-thiosulfoximide **4**.[12] Similarly, the reaction of 1-thioglycerol with nitrosobenzene in the presence of H_2O resulted in the formation of an *N*-phenylsulfoximide.[13]

At this point, the question was addressed which of the two S atoms of **2** would react with H_2O. Because of the electron-withdrawing effect of the formal π-bond in eq 1

$$\mathit{DDAH1}{-}\mathrm{S}{-}\mathrm{N}{=}\mathrm{S}^{+}{-}\mathrm{Hcy} \leftrightarrow \mathit{DDAH1}{-}\mathrm{S}^{+}{=}\mathrm{N}{-}\mathrm{S}{-}\mathrm{Hcy} \quad (1)$$

both S atoms can, in principle, be a target for nucleophilic attack.[14] It should be noted that *N*-thiosulfoximides are stable in water-containing solvents only at low pH.[15] This property is in agreement with the observed pH stability of **4** in MS analysis (Figure 2). The fact that the loss of modification completely recovered the native mass of DDAH-1 indicates that the nucleophilic attack preferentially takes place at Hcy:S according to Scheme 1. A preference of the equilibrium of eq 1 to one site was also observed in the case of asymmetric sulfiliminosulfonium salts.[16] However, in the case of DDAH-1 the strong selectivity for the formation of **4** likely originates from the active-site structure.

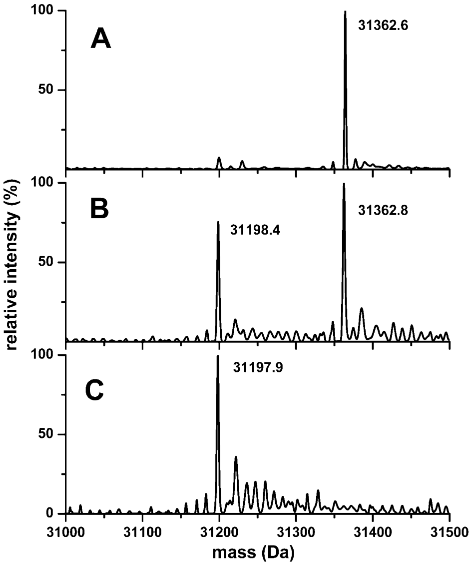


Figure 2. Deconvoluted ESI Q-TOF mass spectra of DDAH-1 incubated with 500 μM HcyNO in 20 mM NH_4OAc/NH_3 (pH 7.4) for 30 min at 37 °C. Samples were injected in (A) 0.1% HCOOH, 50% CH_3CN (pH 2.9), (B) 0.1% HOAc, 50% CH_3OH (pH 3.5), and (C) 20 mM NH_4OAc/NH_3, 50% CH_3CN (pH 9.0).

To conclude, our results indicate that HcyNO forms the covalent dead-end complex **4** with DDAH-1. The formation and stabilization of **4** is accomplished by the protein structure. The results support the recent proposal that the reaction between a thiol and a *S*-nitrosothiol inside a protein cavity may lead to reactions other than transnitrosation.[17] Covalent inhibitors for both currently known mammalian DDAH isoenzymes and their homologue arginine deiminase[3,11] will be widely appreciated in both clinics and research. Thus, HcyNO and the mechanism proposed herein pave the way for the rational design of such inhibitors.

Acknowledgment. We thank Dr. Sergiy Chesnov and Dr. Peter Hunziker for recording the mass spectra. This work was supported by the “Forschungskommission und Nachwuchsförderungskommission der Universität Zürich” (to M.K.), the Swiss National Science Foundation Grant 3100A0-100246/1 (to M.V.), and the “Jubiläumsspende der Universität Zürich” (to M.V.).

Supporting Information Available: Detailed experimental protocols and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Stamler, J. S.; Lamas, S.; Fang, F. C. *Cell* **2001**, *106*, 675−683. (b) Hogg, N. *Annu. Rev. Pharmacol. Toxicol.* **2002**, *42*, 585−600.
(2) (a) Petros, A.; Leone, A.; Moncada, S.; Bennett, D.; Vallance, P. *Cardiovasc. Res.* **1994**, *28*, 34−39. (b) Ashina, M.; Lassen, L. H.; Bendtsen, L.; Jensen, R.; Olesen, J. *Lancet* **1999**, *353*, 287−289. (c). Hobbs, A. J.; Higgs, A.; Moncada, S. *Annu. Rev. Pharmacol. Toxicol.* **1999**, *39*, 191−220.
(3) (a) Vallance, P.; Leiper, J. *Nat. Rev. Drug Discovery* **2002**, *1*, 939−950. (b) Vallance, P. *Fundam. Clin. Pharmacol.* **2003**, *17*, 1−10. (c) Lu, X.; Galkin, A.; Herzberg, O.; Dunaway-Mariano, D. *J. Am. Chem. Soc.* **2004**, *126*, 5374−5375. (d) Magalhães, B. S.; Harris, R.; Plevin, M. J.; Driscoll, P. C. *J. Biomol. Nucl. Magn. Reson.* **2004**, *29*, 463−464. (e) Plevin, M. J.; Magalhães, B. S.; Harris, R.; Sankar, A.; Perkins, S. J.; Driscoll, P. C. *J. Mol. Biol.* **2004**, *341*, 171−184.
(4) (a) MacAllister, R. J.; Parry, H.; Kimoto, M.; Ogawa, T.; Russel, R. J.; Hodson, H.; Whitley, G. St. J.; Vallance, P. *Br. J. Pharmacol.* **1996**, *119*, 1533−1540. (b) Ueda, S.; Kato, S.; Matsuoka, H.; Kimoto, M.; Okuda, S.; Morimatsu, M.; Imaizumi, T. *Circ. Res.* **2003**, *92*, 226−233.
(5) (a) Leiper, J.; Murray-Rust, J.; McDonald, N.; Vallance, P. *Proc. Natl. Acad. Sci. U.S.A.* **2002,** *99*, 13527−13532. (b) Knipp, M.; Braun, O.; Gehrig, P. M.; Sack, R.; Vašák, M. *J. Biol. Chem.* **2003**, *278*, 3410−3416.
(6) To avoid the formation of peroxynitrite all experiments were performed under strictly anaerobic conditions. The interference of redox transition metals was excluded by rendering all solutions metal-free through treatment with Chelex 100 (BioRad) and the addition of 5 mM EDTA.
(7) The product was found stable for at least 5 days at 4 °C. Interestingly, *S*-nitroso-L-cysteine reacted differently. This and the biological implications of our findings will be published elsewhere.
(8) Murray-Rust, J.; Leiper, J.; McAllister, M.; Phelan, J.; Tilley, S.; Santa Maria, J.; Vallance, P.; McDonalds, N. *Nat. Struct. Biol.* **2001**, *8*, 679−683.
(9) (a) Singh, S. P.; Wishnok, J. S.; Keshive, M.; Deen, W. M.; Tannenbaum, S. R. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 14428−14433. (b) Wong, P. S.-Y.; Hyun, J.; Fukuto, J. M.; Shirota, F. N.; DeMaster, E. G.; Shoeman, D. W.; Nagasawa, H. T. *Biochemistry* **1998**, *37*, 5362−5371. (c) Munro, A. P.; Williams, D. L. H. *J. Chem. Soc., Perkin Trans. 2* **2000**, 1794−1797. (d) Wang, K.; Wen, Z.; Zhang, W.; Xian, M.; Cheng, J.-P.; Wang, G. W. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 433−436.
(10) Furukawa, N.; Yoshimura, T.; Oae, S. *Tetrahedron Lett.* **1973**, *23*, 2113−2116.
(11) Galkin, A.; Kulakova, L.; Sarikaya, E.; Lim, K.; Howard, A.; Herzberg, O. *J. Biol. Chem.* **2004**, *279*, 14001−14008.
(12) The proposed mechanism is further supported by the determination of the reaction stoichiometry of 1:1 between DDAH-1 and HcyNO (for details see Supporting Information).
(13) Klehr, H.; Eyer, P.; Schäfer, W. *Biol. Chem. Hoppe-Seyler* **1985**, *336*, 755−760.
(14) Furukawa, N.; Akutagawa, K.; Oae, S. *Phosphorous Sulfur* **1984**, *20*, 1−14.
(15) (a) Oae, S.; Iida, K.; Takata, T. *Phosphorous Sulfur* **1981**, *12*, 103−113. (b) Oae, S.; Akutagawa, K.; Furukawa, N. *Phosphorous Sulfur* **1984**, *19*, 223−234. (c) Yoshimura, T.; Furukawa, N.; Akasaka, T.; Oae, S. *Tetrahedron* **1977**, *33*, 1061−1067.
(16) Nishikawa, Y.; Matsuura, Y.; Kakudo, M.; Akasaka, T.; Fukurawa, N.; Oae, S. *Chem. Lett.* **1978**, 447−450.
(17) Houk, K. N.; Hietbrink, B. N.; Bartberger, M. D.; McCarren, P. R.; Choi, B. Y.; Voyksner, R. D.; Stamler, J. S.; Toone, E. J. *J. Am. Chem. Soc.* **2003**, *125*, 6972−6976.

JA0430200