Inhibition of Human Dimethylarginine Dimethylaminoxydrolyase-1 by S-Nitroso-L-homocysteine and Hydrogen Peroxide

ANALYSIS, QUANTIFICATION, AND IMPLICATIONS FOR HYPERHOMOCYSTEINEMIA

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The plasma concentrations of two cardiovascular risk factors, total homocysteine (tHcy) and asymmetric dimethylarginine (ADMA), correlate with decreased levels of endothelium-derived nitric oxide and subsequent endothelial dysfunction. Homocysteine has been proposed to inhibit the catalytic enzyme of ADMA, dimethylarginine dimethylaminoxydrolyase (DDAH), but the mechanism of this inhibition has not been fully elucidated. Here, the human DDAH isoform-1 (DDAH-1) is heterologously expressed and purified. Cys274 and His173 are identified as active site residues and the pH rate dependence is described. Because oxidation of the active site Cys has been suggested as an inhibitory mechanism in patients with hyperhomocysteinemia, the sensitivity of DDAH-1 to inhibition by L-homocysteine, \( \text{H}_2\text{O}_2 \), and \( S \)-nitroso-L-homocysteine is quantified. DDAH-1 is surprisingly insensitive to inactivation by the powerful oxidant, \( \text{H}_2\text{O}_2 \) (0.088 \( \text{mM} \text{s}^{-1} \text{s}^{-1} \)), possibly because of a substrate-assisted mechanism that allows the active site cysteine to remain predominantly protonated and less reactive in the resting enzyme. In contrast, DDAH-1 is sensitive to inactivation by \( S \)-nitroso-L-homocysteine (3.79 \( \text{mM} \text{s}^{-1} \text{s}^{-1} \)). This work illustrates how a particular catalytic mechanism can result in selective redox regulation and has possible implications for hyperhomocysteinemia.

Endothelium-derived nitric oxide (NO) regulates vasorelaxation as well as other vascular functions (1). Endothelial dysfunction is observed in many conditions including hypercholesterolemia, hypertension, diabetes, hyperhomocysteinemia, chronic renal failure, smoking, and aging, and often correlates with a decrease in endothelium-derived NO and the associated vasodilation (2). Elevated plasma concentrations of at least two cardiovascular risk factors, asymmetric \( N^\text{ε},N^\text{ε} \)-dimethyl-L-arginine (ADMA)\(^2\) and total homocysteine (tHcy), are known to be associated with endothelial dysfunction (3–5). These two markers have been proposed to be mechanistically linked and to mediate at least part of the pathobiology of patients with hyperhomocysteinemia (6–9).

Much of the pathway for inhibition of NO synthesis by ADMA is known. This endogenously produced arginine analog can be transported by the \( \gamma \) cationic amino acid transporter (10) and is known to be an inhibitor of all three NO synthase isoforms (11). Pathological accumulation of ADMA has been proposed as a ubiquitous mechanism for endothelial dysfunction and has even been called an “über marker” for adverse cardiovascular conditions (12). The plasma concentrations of ADMA are partially controlled by one or both isozymes of the enzyme dimethylarginine dimethylaminoxydrolyase (DDAH-1 and DDAH-2), which hydrolyze ADMA (Scheme 1, structure 1) to the non-inhibitory (or less-inhibitory) products citrulline (Scheme 1, structure 2) and dimethylamine (Scheme 1, structure 3) (11). The systemic inhibition of DDAH activity by small molecules (13) and the transgenic overexpression of the DDAH-1 isoform (14) result in respective increases or decreases in blood pressure, as predicted, highlighting the importance of DDAH in controlling ADMA, NO, and vasodilation \( \text{in vivo} \) (15).

The pathway for tHcy inhibition of NO synthesis is less clear. Elevated tHcy levels have been proposed to increase plasma ADMA concentrations by inhibiting DDAH activity through a decrease in expression levels or through direct inhibition by Hcy, by associated reactive oxygen or nitrogen species, or by zinc ions released during oxidative stress (6, 9, 11). Here we report the heterologous expression, purification and characterization of the recombinant human DDAH-1 isoform and quantify its sensitivity to inhibition by L-homocysteine (Hcy), hydrogen peroxide (\( \text{H}_2\text{O}_2 \)), and \( S \)-nitroso-L-homocysteine (HcyNO). The results clearly show that human DDAH-1 is not sensitive to inhibition by physiological concentrations of \( \text{H}_2\text{O}_2 \), despite having an active site cysteine nucleophile. However, DDAH-1 is irreversibly inhibited by HcyNO with sensitivity comparable to typical cysteine-dependent hydrolases. These data suggest that HcyNO may be a biological mediator in the endothelial dysfunction associated with hyperhomocysteinemia.

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* This work was supported in part by Grant RSG-05-061-01-GMC from the American Cancer Society, Grant F-1572 from the Robert A. Welch Foundation, and a seed grant from the Texas Institute for Drug and Diagnostic Development at the University of Texas, Austin. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2. The abbreviations used are: tHcy, total homocysteine; Hcy, homocysteine; HcyNO, \( S \)-nitrosohomocysteine; GSH, glutathione; GSNO, \( S \)-nitrosoglutathione; NO, nitric oxide; BSA, bovine serum albumin; Trx, thioredoxin; DTT, dithio-1,4-threitol; ADMA, asymmetric \( N^\text{ε},N^\text{ε} \)-dimethyl-L-arginine; NMMA, \( N^\text{ε} \)-methyl-L-arginine; SMTc, \( S \)-methyl-thiocitrulline; DDAH, dimethylarginine dimethylaminoxydrolyase; LB, Luria-Bertani; WT, wild-type; ESi, electrospray ionization; MS, mass spectrometry; Ni-NTA, nickel-nitriloacetic acid; MES, 2-(cyclohexylamino)ethanesulfonic acid; CHES, 2-(cyclohexylamino)-1-propanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.
**EXPERIMENTAL PROCEDURES**

**Cloning and Purification of Human DDAH-1**—The coding region for human DDAH-1 (DDAH-1, Protein GI: 6831528) was amplified from an I.M.A.G.E. clone (5189970) obtained from the American Type Culture Collection (ATCC 7273181, Manassas, VA) using two specific end primers: 5′-CTAGCTACGATGCGCCGGGTCACTGACCCACCC-3′ and 5′-CCTAGATCCTCAAGAATGTTTCTTGT-3′. The forward primer contains an NheI restriction site (underlined) followed by 21 bases corresponding to the DDAH-1 coding sequence. The reverse primer contains a BamHI restriction site (underlined) followed by 19 bases complementary to a stop codon and the end of the DDAH-1 coding sequence. Amplification was carried out by the polymerase chain reaction (PCR) using an MJ Research (Waltham, MA) PTC 200 thermal cycler, along with the aforementioned primers, ATCC 7273181, dNTPs, and pfu polymerase in the pfu polymerase buffer (Stratagene, La Jolla, CA) following a temperature program: 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min, followed by a 10-min hold at 72 °C. The PCR-amplified product and the expression vector pET-28a (EMD Biosciences, Carlsbad, CA) were digested with NheI and BamHI restriction enzymes (Invitrogen, Carlsbad, CA) and Qiaquick (Qiagen, Valencia, CA) purified before ligation. The resulting plasmid (pET28-hddah1) was transformed into *Escherichia coli* DH5α cells for amplification, and the insert was sequenced at the DNA Facility (Institute of Molecular and Cellular Biology, The University of Texas) to ensure that there were no undesired mutations. pET28-hddah1 was transformed into *E. coli* BL21 (DE3) cells. Typically, overexpression of DDAH-1 was carried out by inoculating 6 liters of LB medium containing 50 μg/ml kanamycin with 24 ml of inoculant from a saturated overnight culture and shaking at 37 °C. When the culture reached an A₆₀₀ of 0.2, the expression temperature was decreased to 15 °C. Isoproplyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.25 mM when the A₆₀₀ of the culture was between 0.3 and 0.6. The incubation was continued for another 18 h before the cells were harvested by centrifugation at 5,000 × g. The cell pellets from 6 liters of culture were resuspended in 80 ml of lysis buffer (NaP buffer (50 mM Na₂HPO₄, 150 mM NaCl, and 10% glycerol at pH 8.0) supplemented with 5 mM imidazole). The resulting cell suspension was sonicated on ice for 4 min with 30 s burst/rest cycles followed by centrifugation at 34,500 × g for 20 min. The supernatant was incubated with 6 ml of Ni-NTA affinity resin (Qiagen) for 1 h at 4 °C. After harvesting at 1,000 × g, the resin was loaded into a column and washed with 5 column volumes of lysis buffer, followed by 10 column volumes of wash buffer (NaP buffer supplemented with 20 mM imidazole), and finally eluted with 4 column volumes of elution buffer (NaP buffer supplemented with 60 mM imidazole). Fractions containing DDAH-1 were identified by SDS-PAGE and combined. The buffer was exchanged to buffer A (50 mM Na₂HPO₄ at pH 7.0 containing 1.2 mM (NH₄)₂SO₄ and 10% glycerol) using an Amicon Ultra Centrifugal Filter with a 10 kDa molecular weight cutoff (MWCO) (Millipore, Billerica, MA) before the protein solution was applied to a phenyl-Sepharose (GE Healthcare, Piscataway, NJ) hydrophobic column (1.5 × 18 cm). The column was washed with 60 ml of buffer A before the protein was eluted with a linear gradient between 150 ml of buffer A and 150 ml of buffer B (50 mM Na₂HPO₄ at pH 7.0 containing 10% glycerol). The purity of each resulting fraction was examined by SDS-PAGE, and those showing a single DDAH-1 band were pooled. Desalting and concentration were completed with a 10-kDa MWCO Amicon Ultra Centrifugal Filter. The N terminus is distant from the active site (16, 17), so the His₈ tag was left attached. The final purified protein was made 10% in glycerol, flash frozen in N₂ and stored at −80 °C.

**Characterization of DDAH-1**—The molecular weight of purified DDAH-1 was analyzed by electrophoresis on an SDS-PAGE gel. The molecular weight was determined either according to Bradford with bovine serum albumin as the standard or by measuring the absorption at 280 nm. The extinction coefficient of the protein (8,400 M⁻¹ cm⁻¹) was calculated on the basis of the amino acid sequence using Vector NTI, version 10 (Invitrogen). Protein concentrations calculated from the two methods are comparable (within ±10%).

**Site-directed Mutagenesis**—Construction of expression vectors containing mutations at Cys²⁷⁴, Cys²⁷⁵, or His¹⁷³ was achieved using a QuikChange site-directed mutagenesis kit (Stratagene). Three pairs of oligonucleotides: 5′-GGATGGGGCTGCTCACCCGGCCTGCTCATTTTAAAT-3′ and 5′-GTTATTAAACTGACGCGCGTACGCCC-3′; 5′-GATGGGCTGCTCACCCGGCCTGCTCATTTTAAAT-3′ and 5′-CTTTGTTATTTAAACTGACGCGCGTACGCCC-3′; 5′-CCGCTGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAGTGCGGTGCGTTAG
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95 °C for 30 s, followed by 16 cycles of 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 13 min. The reverse primer DpnI was then added to the reaction mixture, which had been cooled down on ice, to digest the parent plasmid. After incubation at 37 °C for 1 h, the remaining mutant plasmid was transformed into E. coli DH5αE competent cells. Amplified plasmid was extracted from a 5-ml E. coli DH5αE culture, and the coding sequence was verified by sequencing. Plasmids with the desired mutations were transformed to E. coli BL21(DE3) cells. Over-expression and purification of the mutant proteins were conducted using the same conditions as described for wild-type DDAH-1.

Steady-state Kinetic Studies—A discontinuous colorimetric ureido derivatization procedure was used to detect the reaction product, citrulline, as described previously (18, 19), and the steady-state catalytic rate constants of DDAH-1 were determined for hydrolysis of Nω,Nω-dimethyl-L-arginine (ADMA), Nω-monomethyl-L-arginine (NMMA), S-methyl-L-thiocitrulline (SMTC), and L-arginine. Typically, various concentrations (10 μM to 10 mM) of each substrate were treated with enzyme (1 μM), all in Na2HPO4 buffer (100 mM) at pH 7.5, 25 °C, and incubated for 30 min before the reaction was quenched with addition of trifluoroacetic acid (0.3 N). Control reactions indicated that citrulline production is linear over this time scale. A control reaction without enzyme was also performed at each substrate concentration, enabling background citrulline levels to be subtracted. A citrulline standard curve (0–400 μM), prepared in the same buffer allowed for quantification of any product formed from 4.5 to 10.6. The buffers used were: at pH 4.5–5.0, sodium acetate (20 mM); at pH 5.5–6.5, MES (20 mM); at pH 6.0–6.5, HEPES (20 mM); at pH 7.0–8.0, HEPES (20 mM); at pH 8.5–10.0, CHES (20 mM); at pH 10.5–10.6, CAPS (50 mM). All buffers contained 100 mM KCl.

pH Dependence of DDAH-1-catalyzed Hydrolysis of ADMA—Steady-state rate constants for DDAH-1 catalyzed hydrolysis of ADMA were determined as described above at pH values ranging from 4.5 to 10.6. The buffers used were: at pH 4.5–5.0, sodium acetate (20 mM); at pH 5.5–6.5, MES (20 mM); at pH 6.0–6.5, HEPES (20 mM); at pH 7.0–8.0, HEPES (20 mM); at pH 8.5–10.0, CHES (20 mM); at pH 10.2–10.6, CAPS (50 mM). All buffers contained 100 mM KCl. Reactions were done at least in duplicate at each pH value, and the observed rates were fit to the Michaelis-Menten equation. All of the fits in this work were completed using either Grafit or KaleidaGraph software (Synergy Software, Reading, PA).

The resulting curves for the pH dependence of both kcat and kcat/Km were fit with a single pKα model (see Equation 1), in which y is substituted by either kcat/Km or kcat, accordingly. The term ymin was fixed at 0.

\[
\log[y_{obs}] = \log[y_{min}] + \frac{(y_{max} - y_{min})}{[1 + 10^{pK_{alpha} - pH}]}
\]  
\[\text{(Eq. 1)}\]

Synthesis of Inhibitors—Because of the short half lives and limited stability of these inhibitors, thiol and nitrosothiol containing compounds were synthesized immediately prior to use. Unless otherwise stated, all of the chemicals were from Sigma-Aldrich.

L-Homocysteine (Scheme 1, structure 1; L-Hcy) was prepared as described previously (18, 19), and the initial rate data were fit directly using a competitive inhibition model (Equation 4) to determine α values, and a linear fit of these α values to Equation 5.

\[
\nu_0 = \frac{V_{max}[S]}{\alpha K_m + [S]}
\]  
\[\text{(Eq. 4)}\]

\[
\alpha = 1 + \frac{[I]}{K_i}
\]  
\[\text{(Eq. 5)}\]
Time- and Concentration-dependent Inhibition of DDAH-1—To monitor any time-dependent inactivation of DDAH-1 activity by \( \text{L-Hcy} \), DDAH-1 (40 \( \mu \)M) was incubated with \( \text{L-Hcy} \) (800 \( \mu \)M) in K\(_2\)HPO\(_4\) buffer (100 mM) at pH 7.5, 37 °C. An aliquot (10 \( \mu \)l) of the preincubation mixture was withdrawn at various time points between 0–30 min and diluted 20-fold into a reaction mixture containing ADMA (0.5 mM) in K\(_2\)HPO\(_4\) buffer (100 mM) at pH 7.5. The resulting reaction mixtures were incubated at 37 °C for 25 min before their citrulline concentrations were determined as described above. To monitor the time-dependent inactivation of DDAH-1 by H\(_2\)O\(_2\), enzyme (40 \( \mu \)M) was incubated with various concentrations of H\(_2\)O\(_2\) (5–25 mM) in K\(_2\)HPO\(_4\) buffer (100 mM) at pH 7.5. Before each experiment, H\(_2\)O\(_2\) was freshly diluted from a concentrated stock (9.78 M, 33%) and quantified using \( \varepsilon_{240\ nm} = 43.6 \text{ M}^{-1} \text{cm}^{-1} \). At various time points between 0 and 20 min, aliquots (10 \( \mu \)l) of the preincubation mix were diluted 20-fold into a reaction mixture containing ADMA (0.1 mM) and 60 units of bovine liver catalase, all in K\(_2\)HPO\(_4\) buffer (100 mM) at pH 7.5. The resulting reaction mixtures were incubated at 37 °C for 25 min before the product concentrations were determined as described above. As an inhibitor-protection experiment, preincubation of DDAH-1 with H\(_2\)O\(_2\) (20 mM) was also repeated in the presence of L-lysine (25 mM). The amounts of catalase and L-lysine included did not interfere with the L-citrulline standard curve. At each H\(_2\)O\(_2\) concentration, remaining DDAH-1 activity was fitted to Equation 6.

\[
\text{Remaining activity} (\%) = 100 \times \exp(-k_{\text{obs}} \times t) + C
\]

(Eq 6)

The observed pseudo first-order inactivation rates, \( k_{\text{obs}} \), were then plotted against the concentrations of H\(_2\)O\(_2\), and the second-order rate constant was obtained as the slope of the resulting linear plot.

To monitor the time-dependent inactivation of DDAH-1 by HcyNO, enzyme (40 \( \mu \)M) was incubated with varying concentrations of freshly prepared HcyNO (0.2–4 mM) in K\(_2\)HPO\(_4\) buffer (100 mM) at pH 7.5. At various time points between 0 and 30 min, aliquots (10 \( \mu \)l) of the preincubation mixture were diluted 20-fold into a reaction mixture containing ADMA (0.5 mM) and K\(_2\)HPO\(_4\) buffer (100 mM) at pH 7.5. The resulting reaction mixtures were incubated at 37 °C for 25 min before the product concentrations were determined. Inhibitor-protection experiments were also conducted by including either L-lysine (25 mM) or \( \text{L-Hcy} \) (1 mM) in preincubation mixtures containing 800 \( \mu \)M HcyNO. The amounts of \( \text{L-Hcy} \) and HcyNO used here do not interfere with the citrulline standard curve. The pseudo first-order inactivation rates and the second-order rate constant for inactivation were obtained as described above.

Reversibility of Inactivation by H\(_2\)O\(_2\) and HcyNO—DDAH-1 (40 \( \mu \)M) was fully inactivated using H\(_2\)O\(_2\) (20 mM) as described above and then treated with bovine liver catalase (100 units) to degrade all remaining H\(_2\)O\(_2\). Subsequent addition of DTT (25 mM) or glutathione (25 mM) to this treated preincubation mix was followed by 20-fold dilution of aliquots (10 \( \mu \)l) at various timepoints (0–20 min) into a reaction mixture containing ADMA (0.1 mM) in K\(_2\)HPO\(_4\) buffer (100 mM) at pH 7.5, which was assayed for remaining DDAH-1 activity as described above. The concentrations of catalase, DTT and glutathione used here did not interfere with citrulline standard curves. In a separate set of experiments, DDAH-1 was fully inactivated using HcyNO and any remaining HcyNO was removed by a size-exclusion spin column (10 kDa MWCO) (Millipore, MA). The preincubation solution was then treated with DTT (5 mM) and recovery of activity was monitored as described above.

RESULTS

Expression, Purification, and Characterization of DDAH-1—Protein expression at 15 °C reduced the formation of inclusion bodies and increased the yield of soluble protein to ~5 mg/liter. The His\(_6\)-tagged DDAH-1 showed only weak binding affinity for Ni-NTA resin and was eluted with relatively low imidazole concentrations (60 mM). Some impurities co-eluted with DDAH-1 and were subsequently removed by hydrophobic phenyl-Sepharose column chromatography. The resulting purified DDAH-1 was homogeneous as gauged by Coomassie-stained SDS-PAGE. Characterization of DDAH-1 by ESI-MS showed a major peak at 33435 ± 10 Da which matches (within error) the mass calculated from the amino acid sequence of His\(_6\)-tagged DDAH-1 after removal of the N-terminal methionine residue (33441 Da). Some preparations also showed a +177 Da peak due to non-enzymic Nα-glucosylation, as described previously (23, 24). Unlike bovine DDAH-1 (25), human DDAH-1 did not co-purify with any bound metal ions (=0.006 equiv) in these experiments. However, zinc is an inhibitor for human DDAH-1.3

Determination of Steady-state Rate Constants—ADMA and NMMA are comparable substrates for wild-type DDAH-1 as reflected in their similar \( k_{\text{cat}}/K_m \) values (Table 1). The \( k_{\text{cat}} \) values for these natural substrates are somewhat slow, but comparable to those reported for other mammalian DDAH enzymes. The artificial substrate SMTc is a better substrate of DDAH-1, with \( k_{\text{cat}}/K_m \) values 10-fold greater than those of ADMA or NMMA. In contrast, turnover of L-arginine, which lacks any terminal methyl groups, was not detected within the limits of our assay.

DDAH belongs to a larger superfamily of enzymes, which share some common mechanistic features (26, 27). Two absolutely conserved residues are an active site Cys and His, which serve as a catalytic nucleophile, and as a general acid/base, respectively. Based on sequence alignments (see below), the active site Cys was predicted to be either Cys\( ^{274} \) or Cys\( ^{275} \), and His to be His\( ^{373} \) in human DDAH-1. Alanine mutations at each of these positions tested their importance to catalysis. C274A and H173A both had no detectable activity, but C275A retained a \( k_{\text{cat}} \) value about half of wild-type protein (Table 1). All mutants behaved similarly to wild-type protein during purification, consistent with a lack of major folding defects in these mutant proteins.

pH Dependence of DDAH-1-catalyzed ADMA Hydrolysis—Although DDAH-1 activity appears to decrease at pH values >10, there is not sufficient data for meaningful fits of the alkaline limb (Fig. 1). Decreases in \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \) values at acidic

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| Source | Variant | Substrate | \(k_{cat}\) \(k_{m}\) | \(k_{cat}/k_{m}\) |
|--------|---------|-----------|----------------|----------------|
| Human | WT | ADMA | 1.66 ± 0.02 | 170 ± 10 | 9.8 ± 0.7 |
|        | NMMA | 0.79 ± 0.02 | 90 ± 10 | 9.2 ± 1 |
|        | SMTC | 1.6 ± 0.2 | 15 ± 5 | 110 ± 50 |
|        | L-Arginine | ND | - | - |
|        | C275A | ADMA | 0.93 ± 0.07 | 350 ± 40 | 27.2 ± 0.5 |
|        | C274A | ADMA | 0.42 ± 0.02 | 190 ± 20 | 22.2 ± 0.3 |
| Human | WT | ADMA | 1.5 | 214* | - |
| Rat | WT | ADMA | 9.2 | 180 | 51 |
|        | NMMA | 5.6 | 360 | 16 |
| Bovine | WT | ADMA | 6.1 ± 0.8 | 810 ± 120 | 8 ± 2 |
|        | Pao | NMMA | 76 ± 2 | 39 ± 9 | 2000 ± 500 |
|        | Pao | ADMA | 35 ± 1 | 44 ± 1 | 800 ± 100 |

* N°°N°°-dimethyl-L-arginine (ADMA); N°°-methyl-L-arginine (NMMA); S-thiocitrulline (SMTCC).

** This study, at pH 7.5 and 24 °C.

* None detected (ND) above background (≤0.008 s⁻¹).

** Calculated from specific activity given in Ref. 51, at pH 6.5, 37 °C; substrate was not specified, but was presumably either ADMA or NMMA.

* Glutathione transferase-DDAH-1 fusion protein (9), at pH 7, 37 °C.

** From Ref. 53, at pH 6.2, 37 °C, using apo protein.

* Not determined.

* From Ref. 52, at pH 6.5, 37 °C.

* Pao stands for P. aeruginosa; from Ref. 39, at pH 8.0, 25 °C.

** Calculated from specific activity given in Ref. 51, at pH 6.5, 37 °C; substrate was not specified, but was presumably either ADMA or NMMA.

** Calculated from specific activity given in Ref. 51, at pH 6.5, 37 °C; substrate was not specified, but was presumably either ADMA or NMMA.

** Not determined.

Conditions are more pronounced and the acidic limbs were fit with apparent \(k_{cat}\) values of 5.3 ± 0.3 and 6.0 ± 0.6, respectively. Notably, both \(k_{cat}\) and \(k_{cat}/k_{m}\) values show a relatively flat pH dependence between 7 and 10, indicating a broad optimal pH range for ADMA hydrolysis.

**Reversible Inhibition by L-Hcy and L-Lysine—** Under our experimental conditions, no time-dependent inactivation of DDAH-1 by L-Hcy was observed during a 20-min incubation period (see below). L-Hcy is a reversible inhibitor of DDAH-1, with an IC₅₀ value (640 ± 50 μM, data not shown) and a calculated \(K_i\) value (300 ± 40 μM) that is similar to the \(K_i\) value reported earlier for competitive inhibition of a recombinant N-glutathione transferase-human DDAH-1 fusion protein by DL-Hcy (333 μM) (9). For easy visual interpretation, a Lineweaver-Burk plot of DDAH-1 inhibition by L-Lys was constructed and shows intersecting lines at \(1/V_{max}\), indicating competitive inhibition (Fig. 2). Non-linear fitting (see “Experimental Procedures”) allowed calculation of the \(K_i\) value for L-Lys (2.8 ± 0.2 mM).

**Time-dependent Inactivation by \(H_2O_2—** Time-dependent inactivation of DDAH-1 by different concentrations of \(H_2O_2\) were fit by Equation 6 to obtain the pseudo first-order inactivation rates, \(k_{inact}\). Secondary plots of these rates against the concentration of \(H_2O_2\) gave a linear plot, yielding a second-order rate constant of 0.088 ± 0.004 M⁻¹ s⁻¹ (Fig. 3). Inactivation of DDAH-1 appears to occur several orders of magnitude slower than \(H_2O_2\) modification of other Cys dependent enzymes known to undergo redox regulation (Table 2). In a “reverse” inhibitor protection experiment, co-incubation with the inhibitor L-Lys actually increases the rate of DDAH-1 inactivation by \(H_2O_2\) (Fig. 4).

**Time-dependent Inactivation by HcyNO—** Time-dependent inactivation of DDAH-1 by different concentrations of HcyNO are fit by Equation 6 (Fig. 5A). A Kitz and Wilson replot of half-life (\(t_{1/2}\)) and the inverse of inactivator concentration (1/[HcyNO]) intersects at the origin (Fig. 5A, inset), indicating that no saturation is observed (28). This observation is consistent with a bimolecular reaction or with a \(k_{inact}\) value much greater than the dissociation rate of the inactivator. Inactivation rates increased linearly with the concentration of HcyNO, consistent with a second-order reaction (Fig. 5B). However, unlike inactivation by \(H_2O_2\), the inactivation rate constant for DDAH-1 by HcyNO (3.79 ± 0.06 M⁻¹ s⁻¹) is on the same order of magnitude as modification of redox-regulated Cys-dependent enzymes by small molecule nitrosothiols (Table 2). Also in contrast to \(H_2O_2\) inactivation, co-incubation with either reversible inhibitor, L-Lys or L-Hcy, protects DDAH-1 against...
inactivation by HcyNO, consistent with inactivation occurring by active site modification (Fig. 6).

Reversibility of Inactivation by H$_2$O$_2$ and HcyNO—DDAH-1 that was fully inactivated by H$_2$O$_2$ was able to recover ~70% activity upon incubation with DTT (Fig. 7). Incubation with the larger thiol GSH did not effectively recover activity. In contrast, treatment with DTT was not able to significantly recover activity from DDAH-1 that had been fully inactivated by HcyNO.

DISCUSSION

Purified recombinant human DDAH-1 displays Michaelis-Menten type kinetics with little change in activity between pH 7 and 10 (Fig. 1). In contrast to the related enzyme human peptidylarginine deiminase-4 (29), human DDAH-1 appears to have a broad optimal pH range. The steady-state rate constants are similar to those determined for DDAH-1 isoforms from rat, cow, and also purified from human liver (Table 1). In general, mammalian DDAH-1 isoforms appear to have similar $K_m$ values but significantly slower $k_{cat}$ values than the bacterial Pseudomonas aeruginosa DDAH (23). These slower turnover rates may reflect the regulatory role of DDAH in controlling basal NO production in mammals as opposed to its putative degradative role in bacteria. In human blood plasma, the concentration of ADMA has been measured in healthy subjects (0.1–2.7 μM) and has also been found to be significantly elevated (0.2–6.0 μM) in a variety of disease states (11). The cytosolic concentration of ADMA in healthy kidney tissue is estimated to be higher (10 μM) than that in plasma (11). Considering these concentrations and the steady-state rate constants (Table 1), human DDAH-1 likely operates under sub-saturating ($k_{cat}/K_m$) conditions in vivo, and thus would be

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\text{FIGURE 3. Time- and concentration-dependent inhibition of DDAH-1 by H$_2$O$_2$. A, time-dependent loss in DDAH activity is observed after preincubation with 5 ( ), 10 ( ), 15 ( ), 20 ( ), and 25 ( ) mM H$_2$O$_2$ with observed inactivation rates of 0.28, 0.58, 1.17, 1.56, and 2.00 \times 10^{-3} \text{s}^{-1}, respectively. Fits are obtained using Equation 6.} \]

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\text{B, observed pseudo-first-order inactivation rates vary linearly with H$_2$O$_2$ concentration and are fit using a second order rate constant of } 0.088 \pm 0.004 \text{ M}^{-1} \text{s}^{-1}. \]

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\text{FIGURE 4. L-Lysine increases DDAH-1 sensitivity to H$_2$O$_2$ inhibition. In comparison to inactivation by 20 mM H$_2$O$_2$ ( ), } 1.56 \times 10^{-3} \text{s}^{-1}, \text{ taken from Fig. 3), incubation with both H$_2$O$_2$ (20 mM) and L-Lys (25 mM) ( ) results in faster observed inactivation rates, as fit to Equation 6. Control incubations of DDAH alone ( ) are stable under these conditions.} \]

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\text{TABLE 2 Inactivation or modification rates by H$_2$O$_2$ and S-nitrosothiols} \]

| Reagent     | Enzyme               | Inactivation/modification rate constant | Ref. |
|-------------|----------------------|----------------------------------------|------|
| H$_2$O$_2$  | Caspase-3            | $750 \pm 14$                           | (54) |
| H$_2$O$_2$  | Cdc25B               | $164 \pm 4$                            | (55) |
| H$_2$O$_2$  | PTP1B                | $43 \pm 4$                             | (56) |
| H$_2$O$_2$  | Glutathione           | $0.87 \pm 0.03$                        | (57) |
| H$_2$O$_2$  | DDAH-1*              | $0.088 \pm 0.004$                      | This study |
| Trx-SNO     | caspase-3            | $196 \pm 4$                            | (58) |
| HcyNO       | DDAH-1*              | $3.79 \pm 0.06$                        | This study |
| GSNO        | Caspase-3            | $1.67 \pm 0.03$                        | (58) |
| GSNO        | PTP1B                | $1.7$                                  | (59) |
| BSA-SNO     | Glutathione*         | $0.02$                                 | (42) |

* pH 7.25, 37 °C. [S] stands for N-acyl-DEVD-7-amino-4-methylcoumarin.
* pH 7.0, 20 °C.
* pH 7.0, 25 °C.
* pH 7.4, 37 °C.
* pH 7.5, 37 °C.
* Trx stands for thioredoxin, pH 7.5, 23 °C.
* BSA-SNO stands for S-nitroso-bovine serum albumin, pH 7.4, 25 °C.
expected to exhibit little selectivity between ADMA and NMMA. Therefore, the specificity of DDAH-1 is not responsible for the 10-fold lower plasma concentrations of NMMA than ADMA typically found in vivo (30). The availability of soluble, active, recombinant human DDAH-1 should now greatly simplify the design and interpretation of anti-tumor (31) drug-screening experiments because previous screens have used non-human enzymes, including bacterial DDAH, bovine DDAH-1, and crude rat kidney homogenates that contain a mixture of DDAH-1 and DDAH-2 isoforms as well as other enzymes that could possibly interfere (13, 32, 33).

Most DDAH-1 isoyme sequences contain a Cys-Cys pair, of which one Cys is presumed to be the catalytic active site nucleophile (Fig. 8). Assigning the active site Cys in human DDAH-1 solely based on sequence alignments is not entirely straightforward because the bovine DDAH-1 structure (94% amino acid identity) lacks this Cys-Cys pair, and because most DDAH-2 sequences appear to have a short sequence insert in this region of the alignment. Here we demonstrate that a C274A mutation in DDAH-1 eliminates catalytic activity, but a C275A mutation only decreases $k_{cat}/K_m$ by 4-fold (Table 1). In addition, muta-
Redox Regulation of DDAH-1

Human DDAH-1 ESKVYKEKDLHMLIVSMSEKVDGLTGDCSCLNLKSVDS
Bovine DDAH-1 ESKVYKEKDLHMLIVSMSEKVDGLTGDCSCLNLKSVDS
Rat DDAH-1 ESKVYKEKDLHMLIVSMSEKVDGLTGDCSCLNLKSVDS
Mouse DDAH-1 ESKVYKEKDLHMLIVSMSEKVDGLTGDCSCLNLKSVDS
Chicken DDAH-1 ESKVYKEKDLHMLIVSMSEKVDGLTGDCSCLNLKSVDS
Human DDAH-2 NSQALQDGDDKTVLSNSKDCSAGSLSLIVSLTPRS
Rat DDAH-2 NSQALQDGDDKTVLSNSKDCSAGSLSLIVSLTPRS
Mouse DDAH-2 NSQALQDGDDKTVLSNSKDCSAGSLSLIVSLTPRS
Pao DDAH RTREIKALGK-YPVIEDTNYKIDGSVOSMLRF-----

FIGURE 8. Partial multiple sequence alignment of DDAH-1 and DDAH-2 isoforms. Residues 244–285 of human DDAH-1 are shown along with the corresponding residues from other DDAH isoforms. Pao stands for P. aeruginosa. The amino acid sequence alignments were constructed using Clustal-W (49) accessed using Biology Workbench (50). Absolutely conserved residues are marked in bold text, and the proposed active site cysteine residues are indicated (*).

Redox Regulation of DDAH-1

Redox Regulation of DDAH-1

Redox Regulation of DDAH-1

Redox Regulation of DDAH-1
contrast to inactivation by H$_2$O$_2$, appears to be irreversible by exogenous thiols (Fig. 8). This observation is consistent with proposing the formation of the same inhibitory N-thio-sulfonamide modification observed with the bovine isoform (17, 33), but the exact chemical mechanism of inactivation of human DDAH-1 by HcyNO is still under study.

The results presented here demonstrate an interesting example in which the catalytic mechanism of DDAH decreases the sensitivity of an active site Cys nucleophile toward oxidation, yet maintains sensitivity to inactivation by a nitrosothiol. The irreversibility and apparent selectivity of human DDAH-1 toward inactivation by HcyNO has possible implications for the endothelial dysfunction associated with hyperhomocysteinemia and supports the hypothesis that this particular nitrosothiol may be a biological mediator.

During review of this manuscript, a crystal structure of recombinant human DDAH-1 showing Cys$^{274}$ and His$^{173}$ as active site residues (our numbering) has been reported (47), as well as the second-order rate constant for inactivation of bovine DDAH-1 by HcyNO ($9 \text{ m}^{-1} \text{s}^{-1}$) (48). These results are very consistent with the human DDAH-1 studies reported herein.

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