Mechanistic Probes of N-Hydroxylation of L-Arginine by the Inducible Nitric Oxide Synthase from Murine Macrophages†

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Received February 6, 1992; Revised Manuscript Received April 17, 1992

ABSTRACT: N^G-Hydroxy-L-arginine, [^{15}N]-N^G-hydroxy-L-arginine, and N^G-hydroxy-N^G-methyl-L-arginine were used as mechanistic probes of the initial step in the reaction catalyzed by nitric oxide synthase isolated from murine macrophages. N^G-Hydroxy-L-arginine was found to be a substrate for nitric oxide synthase with a K_m equal to 28.0 μM, yielding nitric oxide and L-citrulline. NADPH was required for the reaction and (6*R*)-tetrahydro-L-biopterin enhanced the initial rate of nitric oxide formation. The stoichiometry of N^G-hydroxy-L-arginine loss to L-citrulline and nitric oxide (measured as nitrite and nitrate) formation was found to be 1:1:1. N^G-Hydroxy-L-arginine was also observed in small amounts from L-arginine during the enzyme reaction. Studies with [^{15}N]-N^G-hydroxy-L-arginine indicated that the nitrogen in nitric oxide is derived from the oxime nitrogen of [^{15}N]-N^G-hydroxy-L-arginine. N^G-Hydroxy-N^G-methyl-L-arginine was found to be both a reversible and an irreversible inhibitor of nitric oxide synthase, displaying reversible competitive inhibition with K_i equal to 33.5 μM. As an irreversible inhibitor, N^G-hydroxy-N^G-methyl-L-arginine gave k_{inact} equal to 0.16 min^{-1} and K_I equal to 26.5 μM. This inhibition was found to be both time- and concentration-dependent as well as showing substrate protection against inactivation. Gel filtration of an N^G-hydroxy-N^G-methyl-L-arginine-inactivated nitric oxide synthase failed to recover substantial amounts of enzyme activity.

Nitric oxide (•NO)[1] has recently emerged as an important mediator in several key physiological processes. •NO plays seemingly diverse roles in intercellular signaling in the brain (Garthwaite et al., 1988; Bredt & Synder, 1989; Garthwaite, 1991), in the regulation of vascular tone (Palmer et al., 1987, 1988), and in the mediation of cytotoxicity of immunostimulated macrophages (Hibbs et al., 1988; Lancaster & Hibbs, 1990). •NO synthase (NOS; EC 1.14.23) exists in at least two distinct forms: a constitutive Ca^{2+}/calmodulin-dependent form isolated and purified from brain (Bredt & Snyder, 1990; Mayer et al., 1990; Schmidt et al., 1991) and endothelial cells (Pollock et al., 1991) and a cytokine-inducible Ca^{2+}-independent form from rat macrophages (Yui et al., 1991) and murine macrophages (Hevel et al., 1991; Stuehr et al., 1991a). Many other sources of NOS activity have also been found and generally fall into the two categories described above. Although most synthases studied to date have been cytosolic, Pollock et al. (1991) recently reported a particulate form of NOS from endothelial cells. In all cases, the synthesis of •NO occurs through the oxidation of L-arginine to yield L-citrulline, a process which requires both molecular oxygen and NADPH. (6*R*)-Tetrahydro-L-biopterin (BH_4) has been shown to enhance activity of the enzyme from several sources, both constitutive and inducible, including the murine macrophage enzyme (Tayeh & Marletta, 1989; Kwon et al., 1989). In addition, NOS from rat brain (Mayer et al., 1991) and murine macrophages (Hevel et al., 1991; Stuehr et al., 1991a) has been shown to contain both FAD and FMN.

The mechanism of oxidation of L-arginine to •NO is unknown, except for the results from several isotope labeling studies. The reaction is complex as evidenced by the number of cofactors involved. We are involved in determining the mechanism of this oxidation through a number of different avenues, including the use of L-arginine derivatives as chemical probes of reaction intermediates, the processing of alternative substrates, and the design of inhibitors, both reversible and irreversible. As previously proposed by Marletta and coworkers (Iyengar et al., 1987; Marletta et al., 1988), the initial step in the enzymatic conversion of L-arginine to •NO was thought to be N-hydroxylation of the guanidine to yield N^G-hydroxy-L-arginine (L-NHA). We have synthesized both L-NHA and a closely related N^G-alkyl-substituted N^G-hydroxy-L-arginine, N^G-hydroxy-N^G-methyl-L-arginine (L-NHMA), to explore this possibility. Our results show that while N-hydroxylation is indeed the first step in the reaction, subtle modifications of the nitrogen lead to irreversible inactivation of the inducible NOS from murine macrophages.

EXPERIMENTAL PROCEDURES

Materials. N^α-Boc-N^δ-Cbz-L-ornithine was obtained from Bachem, Inc. (Torrance, CA) and used without further purification. Dithiothreitol, Hepes, NADPH, L-arginine, and Sephadex G-25–150 were purchased from Sigma. (6*R*)-5,6,7,8-Tetrahydro-L-biopterin was obtained from Dr. B.

† This research was supported by National Institutes of Health Grant CA 50414. R.A.P. was supported in part by National Institutes of Health Training Grant T32 GM07767.

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[1] Abbreviations: BH_4, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; Boc, *tert*-butyloxycarbonyl; Cbz, carbobenzoxy; $CDCl_3$, deuteriochloroform, CI, chemical ionization; D_2O, deuterium oxide; DSS, desalted 100000*g* supernatant; DTT, dithiothreitol; FAB, fast-atom bombardment; GC/MS, gas chromatography/mass spectrometry; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; L-NHA, N^G-hydroxy-L-arginine; L-NHMA, N^G-hydroxy-N^G-methyl-L-arginine; L-NMA, N^G-methyl-L-arginine; •NO, nitric oxide; NOS, •NO synthase; NO_2^-, nitrite; NO_3^-, nitrate; PCF, pentacyanoaquoferriate; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

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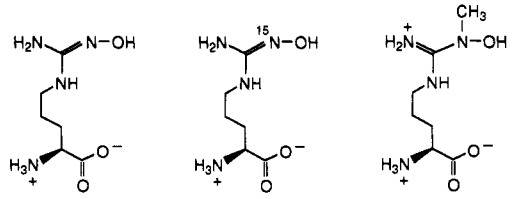

L-NHA [^{15}N]-L-NHA L-NHMA

Schircks Laboratories (Jona, Switzerland). Deuteriochloroform (99.8 atom % D), deuterium oxide (99.9 atom % D), tetramethylsilane (99.9+%, NMR grade), *tert*-butyl alcohol (HPLC grade), trifluoroacetic acid (99+%, spectrophotometric grade), nitrobenzene (ACS grade), hydroxylamine hydrochloride (ACS grade), *N*-methylhydroxylamine hydrochloride, dicyclohexylcarbodiimide, 4-(dimethylamino)pyridine, L-citrulline, and *N*-(1-naphthyl)ethylenediamine dihydrochloride were purchased from Aldrich. $H_2{}^{15}NOH{\cdot}HCl$ (99 atom % ^{15}N) was from Cambridge Isotope Laboratories. [^{15}N]-Nitrobenzene (96.4 atom % ^{15}N) was obtained from MSD Isotopes (St. Louis, MO). Silica gel (230–400 mesh) was from EM Science. Analytical TLC plates (GHL, 250 μm) were purchased from Analtech. Silica Sep-Pak catridges were obtained from Waters. All HPLC solvents were of HPLC grade.

General Methods. All NMR spectra were recorded on a Bruker WM-360. Samples dissolved in $CDCl_3$ are reported in parts per million downfield from tetramethylsilane. ^{13}C-NMR in D_2O used 1,4-dioxane (67.4 ppm) as an internal standard. FAB and CI mass spectra were recorded on a VG Instruments 70-250-S mass spectrometer. GC/MS was done with a Hewlett-Packard 5890 GC with a 5971 mass-selective detector. The GC column was a 12-m HP-1 fused silica capillary with an inner diameter of 0.2 mm. Hydrogenation was carried out on a Parr hydrogenator (Model 3911). TLC plates were visualized by either ninhydrin, PCF (Smith & Horsewell, 1969), or charring with H_2SO_4 (5%) in MeOH. Rotary evaporation was carried out at temperatures no greater than 40 °C using a water aspirator.

Synthesis: (*A*) *N^{α}-Boc-N^{δ}-Cbz-L-Ornithine tert-Butyl Ester.* An ice-cold mixture of N^{α}-Boc-N^{δ}-Cbz-L-ornithine (2.03 g, 5.54 mmol) and dicyclohexylcarbodiimide (1.26 g, 6.09 mmol) in dry CH_2Cl_2 (40 mL) was added to a cold solution of CH_2Cl_2 (20 mL) containing *tert*-butyl alcohol (1.64 g, 22.0 mmol) and 4-(dimethylamino)pyridine (50 mg) with stirring. After being stirred for 2 h at 0 °C, the reaction was allowed to warm to room temperature and proceed for an additional 44 h. The solution was then filtered and concentrated to an oil. Cold anhydrous diethyl ether (30 mL) was added and the resulting precipitate was removed by filtration. After removal of the solvent, the oil was applied to a silica gel column (60 g) and eluted with hexane/ethyl acetate (8:2). Fractions containing the desired compound were combined and concentrated to give 1.36 g (58.0%) of an oil. R_f = 0.68, 3:2 hexane/ethyl acetate. ^{1}H-NMR ($CDCl_3$): 1.43 (s, 9 H), 1.44 (s, 9 H), 1.55–1.91 (m, 4 H), 3.20 (m, 2 H), 4.22 (br s, 1 H), 5.07 (s, 3 H), 5.08–5.19 (br s, 2 H), 7.32 (s, 5 H).

(*B*) *N^{α}-Boc-L-Ornithine tert-Butyl Ester.* N^{α}-Boc-N^{δ}-Cbz-L-ornithine *tert*-butyl ester (1.35 g, 3.20 mmol) was dissolved in MeOH (70 mL) and placed in a Parr bottle flushed with nitrogen. To this solution was added 10% Pd/C (300 mg) in EtOH (15 mL). The bottle was connected to a hydrogenator, flushed several times with H_2, and then pressurized to 40 psi. After being shaken overnight, the solution was filtered through Celite and concentrated to 0.92 g of an oil. TLC of the sample showed one major product along with one minor side product. The sample was not purified further but instead used directly in the next step. R_f = 0.33, 4:1 $CHCl_3$/MeOH. ^{1}H-NMR ($CDCl_3$): 1.44 (s, 9 H), 1.47 (s, 9 H), 1.70–2.00 (m, 6 H), 3.11 (m, 2 H), 4.12 (br s, 1 H), 5.33 (br s, 1 H). ^{13}C-NMR ($CDCl_3$): 23.88, 28.12, 28.46, 29.80, 39.86, 53.93, 80.50, 82.28, 155.88, 171.55.

(*C*) *N^{α}-Boc-N^{δ}-Cyano-L-ornithine tert-Butyl Ester.* An ethereal solution (15 mL) containing cyanogen bromide (0.364 g, 3.43 mmol) was added to an ice-cold solution of N^{α}-Boc-L-ornithine *tert*-butyl ester (0.90 g, 3.12 mmol) in anhydrous Et_2O (40 mL) over a 10-min period. After being stirred for 3 h at 0 °C, the reaction mixture was filtered, concentrated, applied to a silica gel column (40 g), and eluted with $CHCl_3$ to give 0.31 g of an oil. Fractions containing the desired compound were identified by the blue color obtained upon spraying the TLC plate with PCF reagent. Starting material was recovered by eluting with $CHCl_3$/MeOH (9:1). Yield based on recovered starting material was 45.5%. R_f = 0.71, 9:1 $CHCl_3$/MeOH. ^{1}H-NMR ($CDCl_3$): 1.44 (s, 9 H), 1.48 (s, 9 H), 1.60–1.90 (m, 4 H), 3.10 (m, 2 H), 4.14 (br s, 1 H), 5.00 (br s, 1 H), 5.27 (br s, 1 H). ^{13}C-NMR ($CDCl_3$): 25.28, 27.81, 28.15, 29.61, 45.38, 53.29, 79.83, 82.14, 116.43, 155.40, 171.27.

(*D*) *N^{α}-Boc-N^{G}-Hydroxy-L-arginine tert-Butyl Ester.* To a stirring solution of N^{α}-Boc-N^{δ}-cyano-L-ornithine *tert*-butyl ester (0.31 g, 1.00 mmol) in MeOH (15 mL) were added $H_2NOH{\cdot}HCl$ (69.5 mg, 1.10 mmol) and triethylamine (170 μL, 1.20 mmol). The progress of the reaction was followed by reverse-phase HPLC (see below). After 2 h of stirring at room temperature, the mixture was concentrated, applied to a silica gel column (30 g), and eluted with $CHCl_3$/MeOH (9:1). Positive PCF fractions (orange color) were combined and concentrated to dryness by rotary evaporation. The compound was further purified by semipreparative reverse-phase HPLC (see below) to give 0.172 g (49.6%) of pure product. R_f = 0.57, 4:1 $CHCl_3$/MeOH. ^{1}H-NMR ($CDCl_3$): 1.41 (s, 9 H), 1.45 (s, 9 H), 1.55–1.85 (m, 4 H), 3.20 (m, 2 H), 4.08 (br s, 1 H), 5.51 (br s, 1 H), 7.02 (br s, 2 H), 7.54 (br s, 1 H). ^{13}C-NMR ($CDCl_3$): 24.90, 27.90, 28.28, 30.29, 40.86, 53.23, 80.90, 82.86, 156.73, 158.66, 171.70. MS (CI, NH_3) *m/z* (relative intensity): 347 (100, M + 1), 332 (18), 331 (68), 314 (9), 289 (9), 275 (10), 258 (11), 247 (32), 214 (11), 158 (8).

(*E*) *N^{G}-Hydroxy-L-arginine.* Protecting-group hydrolysis of N^{α}-Boc-N^{G}-hydroxy-L-arginine *tert*-butyl ester (0.040 g, 0.12 mmol) was accomplished by stirring with TFA (5 mL) at room temperature for 1.5 h. Removal of the TFA was done by rotary evaporation to yield N^{G}-hydroxy-L-arginine as the trifluoroacetate salt (100% yield based on HPLC). ^{1}H-NMR (D_2O): 1.56–1.82 (m, 4 H), 3.12 (t, 2 H), 3.93 (br, s, 1 H), ^{13}C-NMR (D_2O): 24.58, 27.74, 41.08, 53.41, 115.55 (TFA), 159.57, 163.37 (TFA), 172.58. MS (FAB) *m/z* (relative intensity): 191 (100, M + 1), 175 (42), 158 (6), 149 (14), 128 (22), 116 (16).

(*F*) *N^{α}-Boc-[^{15}N]-N^{G}-Hydroxy-L-arginine tert-Butyl Ester.* This compound was synthesized and purified in an analogous manner to the unlabeled compound except $H_2{}^{15}NOH{\cdot}HCl$ was used. MS (CI, NH_3) *m/z* (relative intensity): 348 (100, M + 1), 332 (27), 331 (27), 314 (32), 289 (19), 275 (39), 258 (36), 248 (32), 232 (31), 214 (32), 158 (23).

(*G*) *[^{15}N]-N^{G}-Hydroxy-L-arginine.* This compound was prepared analogously to the unlabeled compound. MS (FAB) *m/z* (relative intensity): 192 (100, M + 1), 176 (27), 158 (19), 149 (6), 129 (22), 116 (26).

(*H*) *N^{α}-Boc-N^{G}-Hydroxy-N^{G}-methyl-L-arginine tert-Butyl Ester.* *N*-Methylhydroxylamine hydrochloride (0.134 g, 1.60 mmol) and triethylamine (220 μL, 1.60 mmol) were added to a stirring solution of N^{α}-Boc-N^{δ}-cyano-L-ornithine *tert*-butyl ester (0.49 g, 1.56 mmol) in MeOH (10 mL). After being stirred for 2 h at room temperature, the solution was concentrated to dryness, redissolved in methylene chloride (10 mL), and extracted three times (15 mL each) with acidic water (pH 4.5, adjusted with HCl). The combined water extracts were then washed once with methylene chloride (15 mL) followed by removal of the water by rotary evaporation to yield a white foamy solid. For chemical characterization, this solid was further purified by silica gel chromatography (30 g) using $CHCl_3$/MeOH (9:1) to obtain 0.181 g of pure product. R_f = 0.6, 4:1 $CHCl_3$/MeOH. ^{1}H-NMR ($CDCl_3$): 1.42 (s, 9 H), 1.46 (s, 9 H), 1.60–1.79 (m, 4 H), 3.32 (s, 3 H), 3.48 (br s, 2 H), 4.08 (br s, 1 H), 5.43 (br s, 1 H), 6.80 (br s, 1 H), 7.11 (br s, 1 H), 7.40 (br s, 2 H). ^{13}C-NMR (D_2O): 25.17, 28.07, 28.42, 30.18, 40.75, 42.07, 53.42, 80.25, 82.30, 156.19, 157.83, 171.81.

(*I*) *N^{G}-Hydroxy-N^{G}-methyl-L-arginine.* Removal of protecting groups was carried out by stirring N^{a}-Boc-N^{G}-hydroxy-N^{G}-methyl-L-arginine *tert*-butyl ester (0.557 g, 1.55 mmol) in concentrated TFA (6 mL) for 2 h at room temperature, followed by rotary evaporation. The solid was then applied to a 5-mL Dowex AG 50W-X8 cation-exchange resin (200–400 mesh, H^+ form). The resin was first washed with water and then with 0.10 M NH_4OH. Fractions containing the desired compound were combined and concentrated to obtain 0.239 g of pure N^{G}-hydroxy-N^{G}-methyl-L-arginine (75.7% yield). ^{1}H-NMR (D_2O): 1.39–1.60 (m, 4 H), 2.98 (s, 3 H), 2.99 (t, 2 H), 3.40 (t, 1 H). ^{13}C-NMR (D_2O with 1,4-dioxane as internal standard): 25.65, 29.46, 39.45, 41.88, 55.52, 154.33, 177.37. MS (FAB) *m/z* (relative intensity): 205 (100, M + 1), 189 (18.1), 158 (5.9), 142 (5.8), 116 (9.0), 90 (9.5).

Analytical Reverse-Phase HPLC. Reverse-phase HPLC was used to monitor the progress of various reactions. An Altex Ultrasphere ODS column (250 × 4.6 mm, 5 μm) was connected to a Waters HPLC system. Isocratic conditions of 42% CH_3CN (with 0.1% TFA) and 58% H_2O (with 0.1% TFA) were used at a flow rate of 1 mL/min. UV detection (ABI Analytical Kratos Division, Model Spectroflow 783) was used at 229 nm. Under these conditions, the protected *N*-cyano-L-ornithine had a retention time of 12.5 min and the protected N^{G}-hydroxy-L-arginine eluted at 9.5 min.

Semipreparative Reverse-Phase HPLC. N^{α}-Boc-N^{G}-hydroxy-L-arginine *tert*-butyl ester (unlabeled and labeled) was purified by semipreparative reverse-phase HPLC using a Vydac C_{18} column (1.0 × 25 cm, 5 μm, Model 201TP510) under isocratic conditions of 24% CH_3CN (with 0.1% TFA) and 76% H_2O (with 0.1% TFA) with a flow rate of 5 mL/min. The crude mixture was dissolved in MeOH and passed through a Millipore filter (Type FH, 0.5 μm) to remove any particulate matter before injection onto the HPLC. Typically, the retention time was 9.5 min. The fractions containing the compound were combined and the solvent was removed by rotary evaporation.

Purification of •NO Synthase. Cell culture conditions, activation of macrophages, preparation and desalting of the 100000*g* supernatant were carried out as described previously (Tayeh & Marletta, 1989). •NO synthase was purified to homogeneity as previously described (Hevel et al., 1991), and concentrated by centrifugation at 4 °C with Centricon-30 microconcentrators (Amicon Div. of W. R. Grace & Co.-Conn., Beverly, MA). This protein was used in the L-NHMA inactivation experiments as well as the substrate protection experiments. For the K_m determination of L-NHA, a semipurified preparation (approximately 200-fold) was used, which was obtained by chromatography through the 2′,5′-ADP–Sepharose 4B resin according to the published procedure above (except no malate in salt wash).

Protein Determination. The concentration of protein was determined by either the Bradford protein assay or the micro-Bradford protein assay (Bio-Rad) using bovine serum albumin as a standard.

Nitrite and Nitrate Determinations. NO_2^- and NO_3^-, the stable solution decomposition products of •NO, were determined by an automated procedure based on the Greiss reaction as described previously (Green et al., 1982). Samples were passed through a copper-plated cadmium column that reduces NO_3^- to NO_2^-. Total NO_2^- plus NO_3^- was then determined by the reaction of NO_2^- with the Greiss reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride, and 5% H_3PO_4) to form a chromophore absorbing at 543 nm.

Assay of Enzymatic Activity: (*A*) *By Analysis of Nitrite and Nitrate.* The complete assay mixture (300 μL final volume) contained 15 mM Hepes (pH 7.4), 100 μM NADPH (unless otherwise noted), 1 mM $Mg(OAc)_2$, 60 μM BH_4, 1 mM DTT, DSS, and concentrations of L-NHA or L-arginine as indicated in the text. All assays were incubated at 37 °C for 1 h, unless noted otherwise, and analyzed for NO_2^-/NO_3^- levels as described above.

(*B*) *Initial Velocity Measurement via Oxyhemoglobin Assay.* The generation of •NO under initial velocity conditions can be measured utilizing the rapid oxidation of oxyhemoglobin by •NO to produce methemoglobin and NO_3^- as previously described (Olken et al., 1991). The reference cuvette contained 4 μM oxyhemoglobin in 15 mM Hepes (pH 7.4), while the sample cuvette contained 100 μM NADPH, 1 mM $Mg(OAc)_2$, 12 μM BH_4, 167 μM DTT, various L-NHA, L-NHMA, or L-arginine concentrations as specified in each experiment below, an NOS preparation (either DSS, the 2′,5′-ADP–Sepharose eluate, or pure NOS) and 4 μM oxyhemoglobin in 15 mM Hepes (pH 7.4). Assays that contained pure NOS did not contain $Mg(OAc)_2$.

Amino Acid Determination. Amino acid analysis utilizing postcolumn derivatization with ninhydrin was done as previously reported (Tayeh & Marletta, 1989). Samples to be analyzed for L-NHA consumption and L-citrulline formation contained L-NHA at a concentration of 250 μM and were incubated at 37 °C overnight (typically 16 h). Other assay conditions were similar to those used for NO_2^-/NO_3^- assays except NADPH was used at a concentration of 80 μM. Two different eluents (Pickering Laboratories, Mountain View, CA) were used to effect separation of the amino acids. L-Citrulline was analyzed by using a pH 3.28 buffer system, while the other amino acids (L-NHA, L-arginine, and L-ornithine) were separated with a pH 7.40 buffer. Retention times for L-NHA and L-citrulline were 17.8 and 16.5 min, respectively. Standard curves of concentration versus peak area were used to determine the concentrations of the amino acids with L-ornithine used as a standard for L-NHA. The retention time of L-ornithine (14.5 min) and peak shape were very similar to that of L-NHA. When L-arginine was used as a substrate instead of L-NHA, a concentration of 200 μM was used with all other factors remaining the same. Absorbance of amino acid ninhydrin adducts was measured at 546 nm.

Nitrite/Nitrate Production for Determination of ^{15}N Enrichment. The complete assay mixture (1 mL final volume) contained 15 mM Hepes (pH 7.4), 170 μM NADPH, 1 mM $Mg(OAc)_2$, 60 μM BH_4, 1 mM DTT, DSS (1.15 mg of protein) and 2 mM unlabeled [^{14}N]- or labeled [^{15}N]-L-NHA and the reaction was allowed to proceed for 9 h at 37 °C. Samples were deproteinized by filtration through Centricon-10 microconcentrator units prior to the nitration of benzene (see below).

Nitrobenzene Preparation and Analysis. The method of Tesch et al. (1976) as outlined previously (Iyengar et al., 1987) but with the following modifications was used in the preparation of nitrobenzene from the NO_2^- and NO_3^- produced from enzymatic reactions utilizing unlabeled or labeled L-NHA. The deproteinized 1-mL assay containing NO_2^- and NO_3^- was concentrated in a SpeedVac concentrator (Savant, Farmingdale, NY) to approximately 20 μL, followed by the addition of water (130 μL), 0.1 N H_2O_2 (50 μL), concentrated H_2SO_4 (1 mL), and benzene (1 mL). After vigorous shaking for 10 min at room temperature, the benzene layer was removed, passed through a silica Sep-Pak column, and concentrated by a slow stream of nitrogen. Two enzymatic assays (1 mL each) were derivatized and combined to give one nitrobenzene sample. Samples containing either [^{14}N]- or [^{15}N]nitrobenzene were analyzed by GC/MS by selected ion monitoring at m/z 123 and 124, the parent molecular ions of [^{14}N]- and [^{15}N]nitrobenzene, respectively. The injector line was maintained at 240 °C, the transfer line at 280 °C, and the temperature ramp at 60 °C for 0.5 min and then raised by 10 °C/min to 95 °C followed by a hold for 0.5 min. The mass spectrometer was turned off at this point and the GC column was heated to 200 °C. Ions were monitored at m/z of 122, 123, and 124. The retention time for nitrobenzene under these conditions was approximately 3.5 min. The ^{15}N enrichment of the nitrobenzene samples was calculated as previously reported (Green et al., 1982; Biemann, 1962).

L-NHMA Reversible Inhibition Kinetics. The type of reversible inhibition of L-NHMA on NOS was studied under initial rate conditions with the hemoglobin assay. The assay mixtures contained L-arginine (0, 5, 10, 20, 30, or 40 μM), L-NHMA (20, 50, or 100 μM), 100 μM NADPH, 1 mM $Mg(OAc)_2$, 12 μM BH_4, 167 μM DTT, DSS (0.12 mg of protein), and 4 μM oxyhemoglobin in 15 mM Hepes (pH 7.4). Data was analyzed by the method of Hanes and Woolf (Segel, 1975).

Time- and Concentration-Dependent L-NHMA Inactivation Kinetics. The time- and concentration-dependent irreversible inhibition of L-NHMA with pure NOS was studied. Purified NOS (7.5 μg) was added to a preincubation mixture containing L-NHMA (0, 7.5, 10, 20, 40, or 50 μM), NADPH (100 μM), BH_4 (60 μM), DTT (800 μM), and glycerol (20% v/v) in 15 mM Hepes (pH 7.4) in a total volume of 300 μL at 37 °C. Aliquots (50 μL) were removed at specified times (up to 30 min) and reassayed under initial rate conditions by the hemoglobin assay in the presence of L-arginine (2 mM), NADPH (100 μM), BH_4 (12 μM), and DTT (167 μM) in a total volume of 500 μL. Data was analyzed by graphing ln (E/E_0), which is the natural logarithm of the ratio of the remaining enzyme activity at a specified time divided by the initial enzyme activity (time equals zero), versus preincubation time.

Substrate Protection Experiments against L-NHMA Inactivation. Substrate protection experiments using L-arginine or L-NHA against L-NHMA inactivation were also studied with purified NOS. Experiments were carried out under the identical conditions as the L-NHMA inactivation experiments except that preincubation mixtures contained either L-arginine (800 μM) or L-NHA (800 μM). L-NHMA was used at a concentration of 40 μM in both experiments. The results were graphed in the same manner as explained above for the L-NHMA inactivation experiments.

Irreversibility of L-NHMA Inactivation of NOS. A semipurified preparation of NOS, purified through 2′,5′-ADP–Sepharose 4B chromatography according to the published procedure (Hevel et al., 1991) followed by protein concentration by ultrafiltration (Amicon PM10 membrane), was inactivated by incubation with L-NHMA (100 μM), NADPH (100 μM), BH_4 (60 μM), and DTT (800 μM) at 37 °C until the remaining enzyme activity was essentially zero (after 35 min, approximately ≤2% enzyme activity remained). Similarly, a control sample containing NOS, NADPH (100 μM), BH_4 (60 μM), and DTT (800 μM) was incubated under the same conditions as the L-NHMA sample. The L-NHMA-inactivated NOS sample was then passed over a Sephadex G-25–150 column (24 × 0.7 cm) at 4 °C. Likewise, the NOS sample containing cofactors but no substrate or inhibitor was passed over a gel-filtration column. Typically, the majority of the enzyme eluted in one tube, which was resolved from the low molecular weight molecules. For each sample, the protein-containing fraction was then assayed under initial rate conditions to determine remaining enzyme activity.

RESULTS

L-NHA Cofactor Requirements. (A) From Nitrite and Nitrate Results. To determine whether or not L-NHA is a substrate for NOS, L-NHA was added to assays containing DSS and various combinations of cofactors. The amount of NO_2^-/NO_3^- produced after a 1-h incubation at 37 °C was then measured. As shown in Table I, L-NHA was enzymatically turned over to products in the presence of both NADPH and BH_4. The amount of NO_2^-/NO_3^- produced when L-NHA was incubated with DSS with NADPH alone or BH_4 alone was substantially reduced compared to the value obtained when NADPH and BH_4 were used together. When no cofactors were present (i.e., no NADPH or BH_4), NO_2^-/NO_3^- were not formed from L-NHA. Control assays that contained DSS and various cofactors but no L-NHA gave no NO_2^-/NO_3^- production (data not shown). Likewise, assays that contained L-NHA in various combinations with cofactors but no DSS also showed no synthesis of NO_2^-/NO_3^- (data not shown).

(B) From Initial Velocity Production of •NO. The hemoglobin assay was also used to probe the requirements for the enzymatic production of •NO from L-NHA using DSS (Table I). Under initial velocity conditions, •NO was formed in the largest amount when both NADPH and BH_4 were included in the assay. However, when only NADPH was added, the rate of •NO formation was approximately two-thirds that of when both cofactors were used. •NO formation was greatly reduced when BH_4 alone was used (Table I) or insignificant when no cofactors were added. •NO formation seen in the absence of added pterin was also the case when L-arginine was used as a substrate. Both the results from stable end product assays (NO_2^-/NO_3^-) and from initial rate data (HbO_2 assay) show that •NO formation from L-NHA occurs when NADPH alone is present, albeit at a reduced rate.

K_m Determination for L-NHA. To determine the K_m value of L-NHA for NOS, initial rate kinetics were performed via the hemoglobin assay using a semipurified preparation of NOS. The concentration of L-NHA utilized in these assays was varied

Table I: Cofactor Requirements for L-NHA Turnover

assay mixture[a]	NO_2^-/NO_3^- [b] (nmol)	•NO/h[c] (nmol)
control (no NADPH or BH_4)	0	0
NADPH	2.5	14.7
BH_4	1.3	2.5
NADPH and BH_4	8.8	21.2

[a] The control assay mixture contained 15 mM Hepes (pH 7.4), 1 mM $Mg(OAc)_2$, 200 μM L-NHA, and DSS (0.12 mg in column 2, 0.19 in column 3). [b] When the cofactors were added as indicated above, the concentrations used were as follows: 100 μM NADPH and 60 μM BH_4. DTT at a concentration of 1 mM was included in the assays containing BH_4. Incubations were for 1 h at 37 °C as described in Experimental Procedures. [c] When the cofactors were added as indicated above, the concentrations used were as follows: 100 μM NADPH and 12 μM BH_4. DTT at the concentration of 167 μM was included in the assays containing BH_4. The initial rate of •NO formation was followed by the hemoglobin assay as described in Experimental Procedures.

from 6.25 to 100 μM. At concentrations of L-NHA larger than 200 μM, the rate of •NO production was not significantly higher than that obtained for 200 μM (data not shown). Each data point was measured in duplicate and the values used in the K_m determination are an average. The K_m was found to be 28.0 μM as determined by nonlinear regression analysis of the data. The Eadie–Hofstee graph of V versus $V/[S]$ resulted in a value for K_m of 31.4 μM, which is similar to that obtained from the direct fit.

Stoichiometry. The stoichiometry of L-NHA consumption to L-citrulline and NO_2^-/NO_3^- formation was investigated. Analysis of L-NHA loss and L-citrulline production was done by cation-exchange HPLC with postcolumn derivatization using ninhydrin. The amino acids of interest were readily resolved from each other by use of a two-buffer system. The stoichiometry of L-NHA loss to NO_2^-/NO_3^- and L-citrulline formation was found to be 1:1:1 (30.0 ± 4.8, 28.4 ± 2.5, and 28.1 ± 0.5 nmol, respectively; $n = 3$), indicating that one molecule of L-NHA is converted to one molecule of each product, substantiating its intermediacy in the pathway. A control assay containing L-NHA and DSS but no cofactors did not show a substantial amount of citrulline formation under the time course of the assay.

Analysis of •NO Synthase Products from L-Arginine by HPLC. HPLC analysis of the amino acids produced from the NOS oxidation of L-arginine typically resulted in the observation of two distinct products. As expected, the major product of this oxidation was L-citrulline. However, an unknown peak in the chromatogram with a retention time of approximately 18 min was also seen, albeit in small amounts. The retention time for this peak corresponded to that of authentic L-NHA. These results and others from coelution experiments (not shown) strongly suggests that this peak was indeed L-NHA. The small amount of L-NHA that is apparently released, coupled with the fact that no other amino acid intermediate has been observed, suggests that during the catalytic cycle NOS binds arginine and releases only the final products •NO and citrulline.

^{15}N Enrichment of NO_2^-/NO_3^- from [^{15}N]-L-NHA. Analysis of the NO_2^-/NO_3^- produced from unlabeled and labeled L-NHA via its derivatization to nitrobenzene by the acid-catalyzed nitration of benzene provides insight into which nitrogen of L-NHA is further oxidized by the enzyme to yield •NO. When unlabeled L-NHA was used as the •NO synthase substrate, the molecular parent ion of nitrobenzene that was produced had only m/z equal to 123, as is expected, since there is no nitrogen-15 label. Therefore, the percent enrichment in the nitrobenzene was zero. However, when [^{15}N]-L-NHA was utilized as a substrate, an m/z equal to 124 was observed and an enrichment of 94% nitrogen-15 incorporation into nitrobenzene was calculated, indicating that the labeled oxime nitrogen of [^{15}N]-L-NHA is exclusively the one that gives rise to •NO, since the enrichment of [^{15}N]-L-NHA was 93% as determined by mass spectrometry. These results reveal that the further oxidative chemistry to produce •NO required is occurring at the oxime nitrogen.

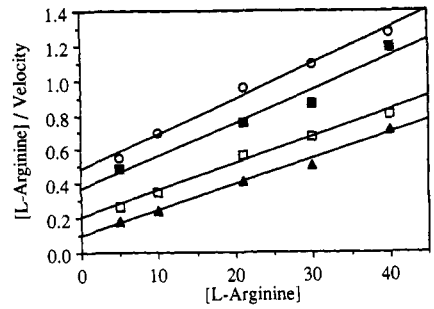

FIGURE 1: Hanes–Woolf analysis of L-NHMA as an inhibitor. Substrate concentration divided by velocity (μM nmol^{-1} h^{-1}) is plotted on the y-axis while substrate concentration (μM) is on the x-axis. The substrate concentration was varied between 5 and 40 μM while L-NHMA concentrations were 0 (▲), 20 (□), 50 (■), and 100 μM (○). See the Experimental Procedures for further assay details.

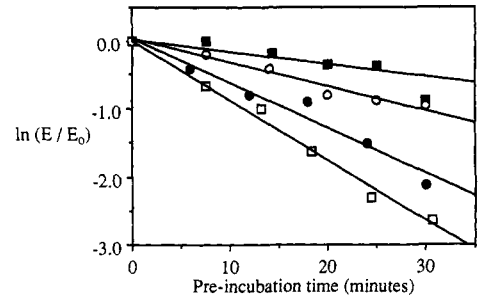

FIGURE 2: Time- and concentration-dependent inactivation of pure NOS by L-NHMA. Assays containing L-NHMA at concentrations of 0 (■), 7.5 (○), 20 (●), or 40 μM (□) were preincubated at 37 °C with pure NOS, NADPH, and BH_4. Aliquots were withdrawn at various time points and reassayed under initial rate conditions in the presence of a saturating level of L-arginine. Data from L-NHMA concentrations of 10 and 50 μM are not shown here. For further details see Experimental Procedures. Data is graphed as ln (E/E_0) vs preincubation time (min). E is the enzyme activity at a specified time and E_0 is the enzyme activity present at time equal to zero.

Reversible Inhibition of NOS by L-NHMA. Due to its structural similarity to L-NHA, an intermediate in the L-arginine to •NO pathway as proven by experiments outlined above and by others, and to its structural similarity to L-NMA, a general inhibitor of NOS, L-NHMA was expected to be a reversible inhibitor of NOS. In fact, this was found to be true. L-NHMA is a reversible competitive inhibitor with a K_i equal to 33.5 μM as determined by Hanes–Woolf analysis (Figure 1).

Irreversible Inhibition of NOS by L-NHMA. When NOS was preincubated at 37 °C with the inhibitor L-NHMA and the cofactors NADPH and BH_4, the enzyme was inactivated at a rate greater than a control sample (which contained no L-NHMA). This inactivation process was found to be both time- and concentration-dependent (Figure 2). In addition, substrate protection was observed when the assays contained either L-arginine or the intermediate L-NHA, indicating that enzyme inactivation is an active-site-directed process (Figure 3). Analysis of the data by the method of Kitz and Wilson (1962) yielded a k_{inact} equal to 0.16 min^{-1} and K_I equal to 26.5 μM (data not shown). This inactivation process could be due to covalent modification of the enzyme at or near the

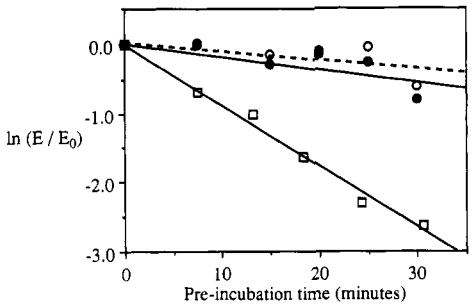

FIGURE 3: Substrate protection of NOS inactivation by L-NHMA. Shown is the protection of NOS inactivation by L-NHMA (40 μM) when the preincubation reaction included L-arginine at 800 μM (●, —), or L-NHA at 800 μM (○, - - -). Also shown for comparison is the rate of inactivation with L-NHMA alone, which has been replotted from Figure 2 (□). Results are graphed as previously described for Figure 2. See Experimental Procedures for details.

active site. The results are also consistent with inhibition caused by the formation of a tight-binding but reversible complex. An L-NHMA-inactivated NOS sample was passed over a gel-filtration column to remove any unbound low molecular weight molecules and the protein-containing fraction was assay for enzyme activity. Under these conditions, approximately 6% of enzyme activity was recovered from the L-NHMA-inactivated enzyme. Therefore, the majority of the enzyme was still irreversibly inhibited after gel filtration.

DISCUSSION

While the mechanism of the reaction catalyzed by NOS has not been completely explained, the results presented above along with previous observations allow for some general conclusions to be reached. Previously we showed that only one of the two chemically equivalent guanidino nitrogens goes on to form NO_2^- and NO_3^- (the solution decomposition products of •NO) and the results obtained in this work clearly show that only the oxime nitrogen of L-NHA exclusively goes on to form •NO. If the other nitrogen of L-NHA was oxidized and went on to form •NO, the ^{15}N-enrichment would have been lower and one would expect *N*-hydroxy-L-citrulline to be formed. This is apparently not the case and is consistent the the observation of others (Stuehr et al., 1991b). While kinetic experiments such as pulse–chase studies have not been carried out, L-NHA appears to be handled similarly to L-arginine and therefore is most likely an intermediate in the reaction. The stoichiometry results, showing that the amount of L-arginine consumed compared to citrulline and NO_2^-/NO_3^- formed was 1:1:1, are also consistent with this conclusion.

As mentioned above, the reaction catalyzed by the •NO synthase involves a number of cofactors. The flavins, FMN and FAD, are relatively tightly bound, and when NOS is isolated by our procedure, the stoichiometry of the flavins to protein is 1:1 (Hevel et al., 1991). The addition of exogenous FAD or FMN to pure NOS has minimal effects on the initial rate of the reaction. However, the reaction is absolutely dependent on NADPH (Marletta et al., 1988) and the dependence of L-NHA on this cofactor provides important mechanistic information as discussed below. The exact function of the pterin is unclear. It is tempting to suggest that the pterin is involved in the hydroxylation step, although an alternative proposal has been put forward (Giovanelli et al., 1991). The pure NOS has been found to contain stoichiometric amounts of bound pterin (Hevel & Marletta, 1992), the significance of which is under study. A relatively tightly bound pterin that has partially fallen off during the purification could be the explanation for the requirement of additional BH_4 to observe maximal initial rate activity.

We studied the cofactor requirements by product analysis (NO_2^- and NO_3^- measurement) and under initial rate conditions (•NO measurement). The results, while qualitatively similar, have important differences. When analyzing for NO_2^- and NO_3^- formation, the rate with NADPH and in the absence of BH_4 is low but significant (Table I). Others have found a similar result (Stuehr et al., 1991b). However, the enzyme is very unstable at 37 °C in the absence of BH_4, and product formation (NO_2^- and NO_3^-) with NADPH alone may simply represent turnover before loss of enzyme activity. Therefore, the dependence on BH_4 appears more pronounced. The initial rate measurements carried out here (Table I) are more meaningful and clearly show that a substantial rate (~70%) of •NO formation is observed in the absence of added BH_4. This represents a true comparison of the cofactor requirements because NOS is fully active during this time. In all cases the highest rate is observed when the two cofactors are present together. The NADPH requirement for L-NHA turnover is consistent with a second hydroxylation reaction in the overall conversion of L-arginine to •NO. Enzymatic conversion of hydroxylamines to nitrones by the microsomal flavin-containing monooxygenase are known (Poulsen et al., 1974). Two successive hydroxylations of L-arginine would represent a four-electron oxidation. After a one-electron oxidation of this intermediate, a number of mechanisms can be written that will lead to the formation of •NO and citrulline.

The results with L-NHMA show that a relatively simple modification of the substrate can lead to irreversible inactivation of the enzyme. We have previously observed that N^G-methyl-L-arginine (L-NMA) irreversibly inactivates macrophage NOS with the characteristics of mechanism-based inhibition (Olken et al., 1991). L-NHMA was synthesized as a probe of further chemistry occurring at the oxime nitrogen. L-NHMA shows time- and concentration-dependent inactivation of NOS, both L-arginine and L-NHA protect against this inactivation, and the inactivation appears to be irreversible. Preliminary results suggest that L-NMA is hydroxylated to L-NHMA (Olken & Marletta, unpublished results) and the k_{inact} and K_I determined for L-NHMA when compared to those constants determined for L-NMA are consistent with the conversion of L-NMA to L-NHMA. We have proposed a mechanism for L-NMA inactivation of NOS (Olken et al., 1991; Olken & Marletta, 1992). However, the studies reported here are consistent with an additional mechanism involving two successive hydroxylations or a hydroxylation followed by a simple two-electron oxidation. Either of these mechanisms would convert L-NMA to a nitrone which might then serve as an electrophilic inactivator of the enzyme. These inactivation mechanisms require further turnover of L-NHMA. While our results are consistent with further processing of L-NHMA before inactivation, the stability of the enzyme in the absence of cofactors precludes a direct answer to that question at this time.

In summary, it is now clear that N-hydroxylation is the first step in the conversion of L-arginine to •NO; however, simple modification of the substrate leads to irreversible inactivation of the enzyme.

ACKNOWLEDGMENT

We thank Joanie Hevel for her experimental expertise and critical comments on the manuscript. We also thank Dr. James Coward (University of Michigan) for use of his Parr hydrogenator and Pete Wishnok (Massachusetts Institute of Technology, S10 RR01901) for the GC/MS analyses. In

addition, we acknowledge Genentech, Inc., for their generous gift of recombinant murine interferon-γ.

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