N^G-Methyl-L-arginine Functions as an Alternate Substrate and Mechanism-Based Inhibitor of Nitric Oxide Synthase†

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Received March 11, 1993; Revised Manuscript Received June 2, 1993•

ABSTRACT: N^G-Methyl-L-arginine (L-NMA) is one of the most commonly used inhibitors of the nitric oxide synthases (NOS). Results reported here demonstrate that L-NMA is an alternate substrate and a mechanism-based inhibitor of the inducible NOS purified from murine macrophages. The irreversible inhibition displays pseudo-first-order kinetics of inactivation with k_{inact} = 0.07 min^{-1} and K_I = 2.7 μM. Inactivation of NOS is enantiospecific for L-NMA, and substrate protection against inactivation is enantiospecific for L-arginine. L-NMA is hydroxylated, producing N^G-hydroxy-N^G-methyl-L-arginine (L-NHMA), and both compounds are slow, partially uncoupled alternate substrates for NOS. Processing of L-NMA by NOS results in four amino acid products: L-NHMA, N^G-hydroxy-L-arginine (L-NHA), L-arginine, and citrulline. Deformylation of L-NMA and L-NHMA precedes the formation of citrulline and nitric oxide (·NO). Partial uncoupling of NADPH oxidation during L-NMA and L-NHMA processing results in hydrogen peroxide formation. The apparent K_m values for L-NMA and L-NHMA are 3.1 and 7.4 μM, respectively. Turnover of L-NMA and L-NHMA to ·NO and citrulline is slow relative to L-arginine: V_{max}(L-arginine)/(L-NMA) = 20:1; V_{max}(L-arginine)/(L-NHMA) = 13:1. NOS contains a functional cytochrome P-450-type heme, and the formation of these products from L-NMA is consistent with cytochrome P-450 monooxygenase chemistry. Other than the NOS reaction intermediate L-NHA, L-NMA and L-NHMA are the first N^G-substituted L-arginines identified as substrates for NOS.

The nitric oxide synthases (NOSs)[1] (E.C. 1.14.13.39) constitute a family of closely related enzymes which synthesize nitric oxide (·NO) and citrulline from L-arginine, O_2, and NADPH (Nathan, 1992; Marletta, 1993). The constitutive cerebellar (Bredt et al., 1991) and endothelial NOSs (Lamas et al., 1992; Janssens et al., 1992; Sessa et al., 1992) and the inducible murine macrophage NOS (Xie et al., 1992; Lyons et al., 1992; Lowenstein et al., 1992) have been cloned and sequenced. Bredt et al. (1991) first reported that the deduced sequence of the cerebellar NOS was homologous to NADPH cytochrome P-450 reductase. Indeed, the P-450 reductase domain of NOS has been found to be highly conserved among all NOS genes sequenced to date. Hevel et al. (1991) reported that NOS contained one tightly bound FAD and FMN each per NOS monomer, analogous to P-450 reductase (Vermilion & Coon, 1978). White and Marletta (1992) subsequently reported that the inducible NOS contained one tightly bound protoporphyrin IX heme per NOS monomer. The reduced carbon monoxide spectrum of this isoform showed a λ_{max} at 447 nm consistent with a P-450-type hemoprotein. Carbon monoxide was found to inhibit citrulline formation, suggesting a functional role for this heme (White & Marletta, 1992). These findings have recently been confirmed (Stuehr & Ikeda-Saito, 1992) and extended to the constitutive NOS isoform (Stuehr & Ikeda-Saito, 1992; McMillan et al., 1992). Thus, the NOS enzymes are the first catalytically self-sufficient P-450s identified in eukaryotes. The only other such P-450 known is P-450_{BM-3}, a fatty acid ω-hydroxylase isolated from *Bacillus megaterium* (Nahri & Fulco, 1986). However, the recent finding that NOS also contains one tightly bound molecule of 6(*R*)-tetrahydro-L-biopterin (H_4B) per active monomer (Hevel & Marletta, 1992) is unique to the NOS family.

The mechanism of NOS may involve successive monooxygenase-like *N*-oxygenations (Figure 1) on of one of the two terminal guanidino nitrogens of L-arginine (Marletta et al., 1988). Consistent with this mechanism, the first monooxygenation product, N^G-hydroxy-L-arginine (L-NHA), has been synthesized and found to undergo oxidation exclusively at the hydroxy-bearing guanidino nitrogen (Stuehr et al., 1991; Pufahl et al., 1992). With L-arginine as the substrate, trace quantities of L-NHA have been detected in solution, which indicates L-NHA remains tightly bound to the active site of NOS during turnover. The substrate specificity of NOS has not been extensively studied. Only L-arginine and L-homoarginine (Hibbs et al., 1987; Iyengar et al., 1987) and the putative NOS reaction intermediate N^G-hydroxy-L-arginine have been shown to support ·NO formation. However, a variety of N^G-monosubstituted L-arginines are potent reversible and irreversible inhibitors of NOS (Olken & Marletta, 1992, and references therein). Of these, N^G-methyl-L-arginine (L-NMA) was the first described (Hibbs et al.,

† This research was supported by USPHS Grants CA 50414 and T32 GM 07767, The American Foundation for Pharmaceutical Education, and The Horace H. Rackham School of Graduate Studies.

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• Abstract published in *Advance ACS Abstracts,* August 15, 1993.

[1] Abbreviations: ·NO, nitric oxide; NOS, nitric oxide synthase; L-NMA, N^G-methyl-L-arginine; L-NHA, N^G-hydroxy-L-arginine; L-NHMA, N^G-hydroxy-N^G-methyl-L-arginine; L-[^{3}H]NMA, N^G-methyl-L-[2,3-^{3}H]arginine; L-[^{14}C] NMA, N^G-[^{14}C]methyl-L-arginine; NO_2^-, nitrite; NO_3^-, nitrate; NO_x^-, the sum of NO_2^- plus NO_3^-; oxyHb, ferrous oxyhemoglobin; metHb, ferric hemoglobin; H_4B, 6(*R*)-tetrahydro-L-biopterin; DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RP-HPLC, reverse-phase high-performance liquid chromatography; t_R, retention time; TLC, thin-layer chromatography; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; HCHO, formaldehyde; H_2O_2, hydrogen peroxide; P-450, cytochrome P-450; SOD, superoxide dismutase.

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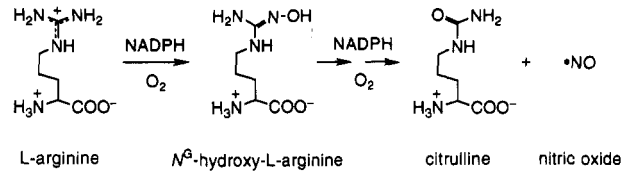


FIGURE 1: Reaction catalyzed by NOS.

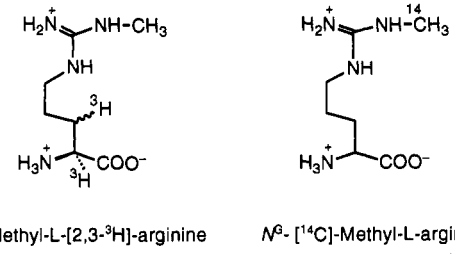


FIGURE 2: Structures of N^G-methyl-L-[2,3-^{3}H]arginine (L-[^{3}H]-NMA) and N^G-[^{14}C]methyl-L-arginine (L-[^{14}C]NMA).

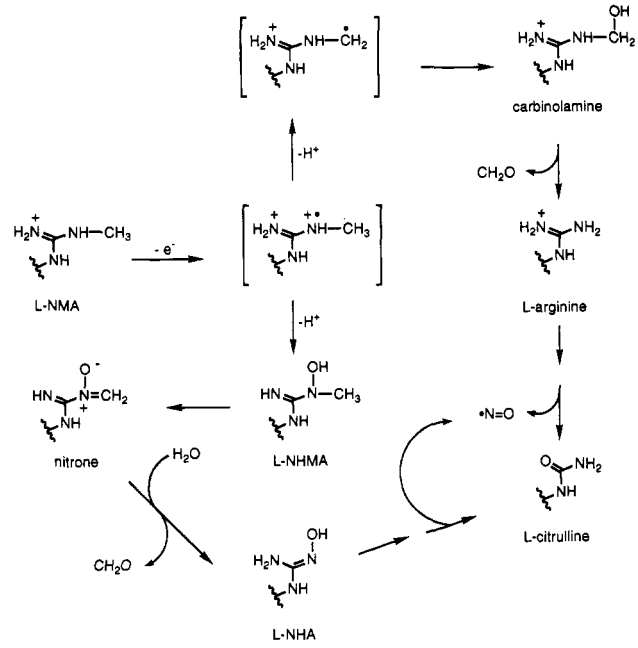
1987) and remains one of the most commonly used inhibitors of neuronal, endothelial, and macrophage NOS (Nathan, 1992; Moncada et al., 1991). In a previous report we characterized N^G-methyl-L-arginine (L-NMA) as a mechanism-based irreversible inhibitor of NOS activity (Olken et al., 1991). A processing scheme in which L-NMA underwent either *N*- or *C*-hydroxylation at the active site of NOS was proposed. We have subsequently described the synthesis of the *N*-oxygenation product, N^G-hydroxy-N^G-methyl-L-arginine (L-NHMA), and found it to be a mechanism-based inhibitor of purified NOS as well (Pufahl et al., 1992). The hypothesis was put forward that L-NHMA underwent further oxidation to a nitrone species which could contribute to the inactivation. In an effort to elucidate the chemical processing of L-NMA at the active site of NOS, N^G-methyl-L-[2,3-^{3}H]arginine (L-[^{3}H]NMA) and N^G-[^{14}C]methyl-L-arginine (L-[^{14}C]NMA) were synthesized (Figure 2). L-NMA was characterized by utilizing these probes, as a slow, partially uncoupled alternate substrate and mechanism-based inhibitor of purified macrophage NOS. L-NHMA was also characterized as a partially uncoupled alternate substrate of NOS. We propose in Scheme I a pathway for L-NMA and L-NHMA processing which is consistent with the results presented in this report.

MATERIALS AND METHODS

Materials. Oxyhemoglobin (human A_o, ferrous), superoxide dismutase (bovine liver), catalase (bovine erythrocytes), D-NMA, D-arginine, nitrate reductase (*Aspergillus* sp.), L-glutamic dehydrogenase (*Candida utilus*), and bovine serum albumin were purchased from Sigma (St. Louis, MO). Dowex AG 50W-X8 [H^+] and Dowex AG 1-X8 [Cl^-] were purchased from Bio-Rad (Richmond, CA). 6(*R*)-5,6,7,8-Tetrahydro-L-biopterin was purchased from Dr. B. Schircks Laboratories (Jona, Switzerland). Cation-exchange HPLC buffers (pH 3.28 and 7.40 eluents and pH 2.2 diluent) were purchased from Pickering (Mountain View, CA). Flow-scint II and III were purchased from Packard (Downers Grove, IL). AquaSil siliconizing fluid and Fluoraldehyde reagent were purchased from Pierce (Rockford, IL). L-[2,3-^{3}H]Ornithine, $^{14}CH_3NH_2$·HCl, and [^{14}C]HCHO were purchased from NEN-Du Pont (Wilmington, DE). L-[2,3,4,5-^{3}H]Arginine was purchased from Amersham (Arlington Heights, IL). L-NHA and L-NHMA were synthesized as described (Pufahl et al., 1992). All other reagents were of the highest grade commercially available.

General Methods and Procedures. Rotary evaporation was carried out at temperatures no greater than 40 °C. All assays or reactions were initiated with NOS unless otherwise noted. The concentrations of stock solutions of NADPH were determined spectrophotometrically (ϵ_{340} = 6220 M^{-1} cm^{-1}) immediately prior to use. Protein concentration was determined with the Micro BCA kit (Pierce, Inc.) using bovine serum albumin as a standard. Liquid scintillation counting was carried out on a Beckman LS 1801 liquid scintillation counter. RAW 264.7 murine macrophages were grown, activated, and harvested as described (Tayeh & Marletta, 1989). NOS was purified as described (White & Marletta, 1992). K_m values were determined with a nonlinear, least-squares fit of the data using the KaleidaGraph program (Synergy Software, Reading, PA). Other plots were fit by linear regression.

Scheme I: Proposed Processing of L-NMA by NOS[a]



[a] The enzyme-dependent formation of the amino acids L-NHMA, L-arginine, L-NHA, and citrulline, as well as ·NO, HCHO, and H_2O_2 (not shown), has been demonstrated (see text). The formation of the carbinolamine (via *C*-hydroxylation) and the nitrone (via a second *N*-hydroxylation and subsequent dehydration) are proposed, but were not detected. The stability of these proposed intermediates in neutral aqueous solution is not known.

Analytical TLC of Radioactive Samples. Analytical TLC of radioactive samples was carried out on 5 × 20-cm strips of aluminum-backed silica gel 60F-250. Amino acid standards of L-citrulline (R_f = 0.8), L-NHMA (R_f = 0.6), L-NMA (R_f = 0.4), L-arginine (R_f = 0.3), and L-ornithine (R_f = 0.2) were either cospotted with samples or spotted separately and were developed with 2:2:1 (v/v/v) $CHCl_3$/MeOH/NH_4OH and visualized with a ninhydrin spray reagent. The TLC plate was cut into 1-cm sections, the silica gel was scraped from the backing, and the radioactivity was measured by liquid scintillation counting.

Amino Acid Analysis by Analytical Strong-Cation-Exchange HPLC with Radiochemical Detection. Samples to be analyzed (25-150 μL) were dissolved with pH 2.2 diluent buffer sufficient to produce a total volume of 210 μL. Samples (200 μL) were analyzed by HPLC using a Pickering strong-cation-exchange column (4.6 × 250-mm, Na^+) which was heated to 45 °C. The mobile-phase flow rate was 0.3 mL min^{-1}. Elution of amino acids was performed with a step gradient of pH 3.40 buffer for 34 min followed by pH 7.40

buffer for 76 min. Radiochemical detection was carried out with a Radiomatic A-200 (Radiomatic, Tampa, FL) equipped with a 500-μL TRLSC flow cell, utilizing a 4:1 ratio of Flow Scint III scintillant to HPLC eluent.

Amino Acid Analysis by Analytical Reverse-Phase HPLC with Fluorescence Detection. Samples (15 μL) were combined at 22 °C with 15 μL of Fluoraldehyde reagent for 1 min. Aliquots (20 μL) were analyzed by HPLC using a Microsorb AAAnalysis, type O column (4.6 × 100 mm) fitted with a guard column (4.6 × 150 mm). The mobile phase was a linear gradient from 100% buffer A (95% 0.1 M NaOAc, pH 7.2, 4.5% methanol, and 0.5% THF) and 0% buffer B (methanol) to 20% buffer A and 80% buffer B over 45 min. A Waters 470 scanning fluorescence detector (λ_{ex} = 340 nm; λ_{em} = 440 nm) was used in conjunction with a Hewlett-Packard 3396A integrator. The sensitivity of detection varied between amino acids, but was generally less than 10 pmol per injection.

Initial Velocity Measurements via the Hemoglobin Assay. The generation of ·NO by NOS was measured by utilizig the rapid oxidation of oxyHb to metHb by ·NO as described (Olken et al., 1991), with the following modifications. The reference cuvette contained 500 μL of 15 mM Hepes, pH 7.4. Assay cuvettes contained purified NOS, 100 or 200 μM NADPH, 70 μM DTT, 6 μM H_4B, 4 μM oxyHb, and substrate, diluted to a total volume of 500 μL with 15 mM Hepes, pH 7.4. Certain assays also contained 150 units of SOD and 100 units of catalase. All assays were performed at 37 °C on a Perkin Elmer (Norwalk, CT) 553 Fast Scan UV/vis spectrophotometer equipped with a Perkin-Elmer R100 chart recorder.

Synthesis of N^G-Methyl-L-arginine. L-NMA was synthesized (Olken & Marletta, 1992) and purified as the flavianic acid salt as described (Lambert et al., 1992). Flavianate was exchanged for chloride by stirring with Dowex AG 1-X8 as described (Cho et al., 1984). The free base of L-NMA was obtained and converted to the acetate salt as described (Patthy et al., 1977). Elemental analysis of the monoflavinate and monoacetate salts and RP-HPLC of the acetate salt indicated that the products were analytically pure.

Synthesis of N^G-Methyl-L-[2,3-^{3}H]arginine. L-[^{3}H]NMA (specific activity 33.4 mCi/mmol) was synthesized from L-[2,3-^{3}H]ornithine (250 μCi, 46.5 Ci/mmol) by reaction with *N,S*-dimethylpseudothiouronium iodide. L-[^{3}H]Ornithine was supplied in 250 μL of an acidified ethanolic solution. This solution was transferred to a 300-μL V-vial (Wheaton, Millville, NJ) and frozen, and the solvent was removed by sublimation. The V-vial was carefully transferred into a fume hood, a spin vane was added, and 7.1 μmol (20 μL) of L-ornithine was added. *N,S*-Dimethylpseudothiouronium iodide (32 μmol) was added in one portion to the stirring solution. The V-vial was sealed with a Teflon-lined cap and vented with a needle to permit the escape of methanethiol. One additional aliquot (20 μL) of distilled water was added after 2 days to maintain the volume of the solution. The reaction was stirred for 3 days at room temperature. The reaction mixture was spotted onto two separate 20 × 20-cm 1000 μm silica gel G glass-backed TLC plates and developed with 2:2:1 (v/v/v) $CHCl_3$/MeOH/NH_4OH. Since unreacted L-[^{3}H]ornithine and the product L-[^{3}H]NMA run closely in this solvent system, the TLC plates were developed twice to maximize the separation. The positions of L-NMA standards which had been spotted onto the margins of each TLC plate were visualized with ninhydrin. The silica gel in the L-NMA zone of each plate (R_f = 0.5–0.7) was scraped, and N^G-methyl-L-[2,3-^{3}H]NMA (59 μCi, 24% radiochemical yield) was eluted with water. Strong-cation-exchange HPLC analysis with radiochemical detection demonstrated that the L-[^{3}H]NMA was 90.8% radiochemically pure. The radiochemical impurities consisted of several percent each of L-[^{3}H]ornithine, [^{3}H]citrulline, and tritium which eluted in the void volume.

Synthesis of N^G-[^{14}C]Methyl-L-arginine. (i) N-Benzoyl-N′-[^{14}C]methylthiourea. [^{14}C]Methylamine HCl (250 μCi, 55.1 mCi/mmol, 4.5 μmol) was dissolved in the shipping vial into 100 μL of water and 20 μL of acetone. This was transferred into a 300-μL V-vial equipped with a spin vane. A second 50-μL rinsing of the stock vial with acetone was also added to the V-vial. To this was added 22 μmol of benzoyl isothiocyanate and 22 μmol of triethylamine. This was allowed to stir at room temperature for 1 h.

(ii) N-[^{14}C]Methylthiourea. The reaction mixture containing *N*-benzoyl-*N*′-[^{14}C]methylthiourea was transferred into a 5-mL V-vial equipped with a spin vane. To this was added 1 mL of 1 N NaOH, and the solution was heated to 65–75 °C for 25 min. The reaction mixture was cooled in an ice bath and acidified with an excess of 4 N HCl. The pH was adjusted to 4.5 with 1 N NH_4OH. The solution was diluted with water to a total volume of 10 mL and applied to a 10-mL column of Dowex AG 50W-X8 [H^+] to remove unreacted $^{14}CH_3NH_3^+$. The resin was washed with 50 mL of water, and the 60-mL flow-through fraction was concentrated by rotary evaporation.

(iii) N-[^{14}C]Methyl-S-methylpseudothiouronium Iodide. Crude *N*-[^{14}C]methylthiourea was transferred into a 25-mL pear-shaped flask equipped with a magnetic stir bar and a reflux condensor was and redissolved in 5 mL of acetone. Iodomethane (5.4 mmol) was added dropwise at room temperature. This was heated to 60–65 °C in an oil bath for 20 min.

(iv) N^G-[^{14}C]Methyl-L-arginine. The solution containing *N*-[^{14}C]methyl-*S*-methylpseudothiouronium iodide was concentrated to dryness by rotary evaporation. The residue was resolubilized with 300 μL of water and 400 μL of acetone and transferred to a 1-mL V-vial. To this was added 100 μmol of L-ornithine. The acetone was allowed to evaporate over 2 days at room temperature. The remaining 400 μL of reaction solution was heated to 80–90 °C for 5 h. TLC indicated that approximately 50% the radioactivity was present as L-[^{14}C]-NMA, while the other 50% migrated at the solvent front. The reaction solution (150 μL) was spotted onto two 20 × 20-cm 250-μm silica gel G TLC plates. L-NMA standards were spotted at either end of these two plates. The two plates were developed with 2:2:1 (v/v/v) $CHCl_3$/MeOH/NH_4OH. The silica in the L-NMA zone of each plate (R_f = 0.3–0.5) was scraped, and L-[^{14}C]NMA (20 μCi, 8% radiochemical yield) was eluted with water. Strong-cation-exchange HPLC with radiochemical detection demonstrated that the L-[^{14}C]NMA was 97.3% radiochemically pure.

Enzymatic Synthesis of [^{3}H]Citrulline. A reaction mixture containing 3 mg of desalted 100000g supernatant (Tayeh & Marletta, 1989), 250 μM NADPH, 60 μM H_4B, and 15 μCi of L-[^{3}H]arginine (62 Ci/mmol) was allowed to react at 37 °C. [^{3}H]Citrulline was isolated by chromatography over Dowex AG 50W-X8 [Na^+]. Strong-cation-exchange HPLC with radiochemical detection demonstrated the product to be ≥95% radiochemically pure.

Identification of Amino Acid Products Derived from Excess[2] L-NMA or L-NHMA by RP-HPLC. Reaction mixtures containing 15 μg of purified NOS, 250 μM NADPH, 170 μM DTT, 14 μM H_4B, and 50 μM L-NMA or L-NHMA in 15 mM Hepes, pH 7.4, in a total volume of 500 μL were allowed to react at 37 °C for 1 h. Samples were analyzed for amino acid content by RP-HPLC. Identification of amino acids was established by the characteristic retention time of each amino acid and by co-injection of samples with amino acid standards.

Amino Acid Analysis via HPLC or TLC of Incubation Mixtures with Limited[2] L-NMA, L-[^{14}C]NMA, or L-[^{3}H]-NMA. Incubation mixtures of 10 μM L-NMA, 12 μM L-[^{3}H]-NMA, or 7 μM L-[^{14}C]NMA and 10 μg of NOS, 350 μM DTT, 200 μM NADPH, and 30 μM H_4B in 15 mM Hepes, pH 7.4, in a total volume of 500 μL, were allowed to react for 30 min at 37 °C. Incubations were analyzed by RP-HPLC, cation exchange HPLC, or analytical TLC as described.

Identification of [^{14}C]HCHO as a Reaction Product from L-[^{14}C]NMA. A reaction mixture of 70 μg of purified NOS, 700 μM DTT, 250 μM NADPH, 60 μM H_4B, and 275 μM L-[^{14}C]NMA (3.3 mCi/mmol) in 15 mM Hepes, pH 7.4, in a total volume of 1.5 mL was allowed to react at 37 °C for 1 h. Aliquots (24 μL) of the incubation mixture were combined with 1 μL of a 1:6 dilution (in acetonitrile) of Brady's reagent (3 g of 2,4-dinitrophenylhydrazine dissolved in 15 mL of concentrated sulfuric acid and diluted into 70 mL of 95% EtOH and 20 mL of water) and allowed to react at room temperature for 8 min. The samples (20 μL) were analyzed by HPLC using a Spherosorb C-18 (LDC Analytical) reverse-phase column (4.6 × 250 mm). The mobile phase was an isocratic mixture of 60% acetonitrile and 40% water run at 1 mL min^{-1}. The HPLC eluate was plumbed into a stream splitter built into a Radiomatic A-200 radiochemical detector. The splitter (cycle time 2 s) diverted 50% of the HPLC eluate into a Waters 440 absorbance detector fitted with a 254-nm filter and 50% into the radiochemical detector equipped with a 500-μL TRLSC flow cell. Flow Scint II was used with a 5:1 ratio of scintillant to HPLC eluate. The authentic 2,4-dinitrophenylhydrazone of HCHO was prepared by combining equal volumes (1 mL) of reagent grade HCHO and freshly prepared Brady's reagent. The yellow precipitate was filtered, washed with cold water, and crystallized from 95% ethanol [mp 166.5–167 °C; lit. 166 °C (Shriner et al., 1980)].

Stoichiometry of [^{3}H]Citrulline to NO_x^- Formation from L-[^{3}H]NMA. Assay mixtures contained purified NOS (7–30 μg), 500 μM NADPH, 350 μM DTT, 30 μM H_4B, and 103 μM L-[^{3}H]NMA (1.06 mCi/mmol) in 15 mM Hepes, pH 7.4, in a total volume of 700 μL. Incubations proceeded at 37 °C for 1.5 h. [^{3}H]Citrulline and NO_x^- were measured as described below.

[^{3}H]Citrulline and [^{14}C]HCHO Determinations. Aliquots of reaction mixtures were applied to 1-mL columns of Dowex AG 50W-X8 [Na^+]. [^{3}H]Citrulline and [^{14}C]HCHO pass through the resin and were eluted with 3 mL of water directly into 15 mL of scintillant. RP-HPLC analysis demonstrated that citrulline was the sole amino acid formed from L-NMA which passed through 1 mL of Dowex AG 50W-X8 [Na^+]. Assays for [^{3}H]citrulline were corrected for recovery of [^{3}H]citrulline (≥97%). The recovery of [^{14}C]HCHO from incubation mixtures was determined to be 100%.

NO_2^- and NO_3^- Determinations. The sum of NO_2^- and NO_3^- (NO_x^-) was measured as NO_2^- via the Griess reaction as follows. Aliquots (210 μL) of sample were incubated for 30 min at 22 °C with 60 milliunits of nitrate reductase and 25 μM additional NADPH. Excess NADPH was consumed by incubation of the sample for 10 min at 22 °C with 200 milliunits of L-glutamic dehydrogenase, 100 mM NH_4Cl, and 4 mM freshly prepared α-ketoglutarate. Under these conditions NO_3^- is quantitatively converted to NO_2^-, and the residual NADPH is oxidized to $NADP^+$. Freshly prepared Griess reagent (1% sulfanilamide, 0.1% (naphthyl)ethylenediamine dihydrochloride, and 5% $H_3PO_4^-$) was added, and the sample (500 μL total volume) was allowed to react at 37 °C for 5 min. Absorbances were recorded at 543 nm vs a reagent blank which contained buffer and Greiss reagent.

Stoichiometry of [^{14}C]HCHO to [^{3}H]Citrulline Formation from ^{3}H- and ^{14}C-Labeled L-NMA. The stoichiometry of H^{14}CHO to [^{3}H]citrulline formation was investigated with excess[2] L-NMA. Incubation mixtures contained 5–25 μg of NOS, 700 μM DTT, 250 μM NADPH, 250 μM ^{3}H- and ^{14}C-labeled L-NMA (0.73 and 0.19 mCi/mmol, respectively), and 60 μM H_4B in 15 mM Hepes (10% glycerol, v/v), pH 7.4, in a final volume of 1.0 mL. Reactions proceeded at 37 °C for 1 h. Aliquots (400 μL) were applied to 1 mL of Dowex AG 50W-X8 [Na^+]. [^{3}H]Citrulline and [^{14}C]HCHO were eluted with 3 mL of water into 15 mL of scintillant. Samples were analyzed for tritium (citrulline) and carbon-14 (HCHO) simultaneously by liquid scintillation counting with dual-label detection. The stoichiometry of [^{14}C]HCHO to [^{3}H]citrulline formation was also investigated with limited[2] L-NMA. These experiments were done as described above, with the exception that the concentration of ^{3}H- or ^{14}C-labeled L-NMA was 10 or 12 μM, respectively.

Stoichiometry of NADPH Consumption to [^{3}H]Citrulline Formation for L-[^{3}H]NMA. Incubation mixtures contained 6 μg of NOS, 700 μM DTT, 60 μM H_4B, 0–50 μM NADPH, and 40 μM L-[^{3}H]NMA (3.92 mCi/mmol) in 15 mM Hepes, pH 7.4, in a total volume of 500 μL. Incubations were initiated with NOS and proceeded at 37 °C for 25 min. Samples were analyzed for [^{3}H]citrulline as described.

Stoichiometry of H_2O_2 Formation to NADPH Oxidation for L-NMA or L-NHMA. Incubations contained 5 μg of NOS, 40 μm L-NMA or 100 μM L-NHMA, and 0–50 μM NADPH in 15 mM Hepes, pH 7.4, in a total volume of 500 μL. Following incubation at 37 °C for 25 min, the samples were placed on ice. Assays for peroxide were carried out as described (Hildebrant et al., 1978). Aliquots (210 μL) of the sample were combined with 5 μL of 6 N TCA, 180 μL of 10.2 mM ferrous ammonium sulfate, and 65 μL of 2.6 M potassium thiocyanate. This was mixed gently and allowed to stand at room temperature for 5 min. Samples were read at 474 nm vs a blank which contained buffer and the reagents for the peroxide assay. The concentration of H_2O_2 in stock solution was determined spectrophotometrically immediately prior to use (ϵ_{240} = 43.6 M^{-1} cm^{-1}).

Rate of [^{3}H]Citrulline Generation from L-[^{3}H]NMA or L-[^{3}H]Arginine by NOS in the Presence or Absence of SOD

[2] The partition ratio of L-NMA is approximately 100. Therefore, NOS processes significant amounts of L-NMA prior to enzyme inactivation. Most experiments reported herein were carried out with excess L-NMA at concentrations such that 20% or less would be processed prior to NOS inactivation. In these experiments L-NMA remained the most prevalent amino acid present in solution, effectively precluding further reaction of L-NHA, L-arginine, or L-NHMA released from the active site of NOS. Certain experiments deliberately utilized concentrations of L-NMA insufficient to inactivate all NOS present. In these experiments with limited L-NMA, the processing of L-NMA and amino acid products derived therefrom could continue to completion. The terms "excess" and "limited" L-NMA refer to these two distinct types of experimental conditions.

and Catalase. Purified NOS (5 μg) was combined with 200 μM NADPH, 70 μM DTT, and 6 μM H_4B in 15 mM Hepes, pH 7.4. Some assays contained 150 units of SOD and 100 units of catalase. Incubations were diluted to 490 μL with 15 mM Hepes, pH 7.4, and allowed to preequilibrate at 37 °C for 3 min. Reactions were initiated with 100 μM L-[^{3}H]-arginine (1.95 mCi/mmol) or 40 μM L-[^{3}H]NMA (3.75 mCi/mmol). After 5 min the reactions were quickly frozen at −80 °C. Aliquots (400 μL) were analyzed for [^{3}H]citrulline as described above.

K_m Determinations for L-NMA and L-NHMA. Assay mixtures contained purified NOS (3 μg), 200 μM NADPH, 150 units of SOD, 100 units of catalase, 70 μM DTT, 6 μM H_4B, 4 μM oxyHb, and substrate in 15 mM Hepes, pH 7.4, in a total volume of 500 μL. Assays contained 0–25 μM L-NMA or 0–100 μM L-NHMA.

NADPH Dependence of L-NMA and L-NHMA Processing by NOS. The NADPH dependence of L-NMA and L-NHMA processing by NOS was investigated by amino acid analysis via RP-HPLC. Purified NOS (10 μg) was combined with 700 μM DTT, 60 μM H_4B, and 1 mM L-NMA or 100 μM L-NHMA with or without 100 μM NADPH in 15 mM Hepes, pH 7.4, in a total volume of 500 μL. Incubations proceeded for 1 h at 37 °C and were analyzed by RP-HPLC as described.

Kinetics of Irreversible Inhibition of NOS Activity. Purified NOS (2 μg), 700 μM DTT, 100 μM NADPH, 60 μM H_4B, and 2.0–100 μM L-NMA or 100 μM D-NMA were combined in 15 mM Hepes (20% glycerol, v/v), pH 7.4, in a total volume of 300 μL and preincubated at 37 °C. Aliquots (50 μL) were removed at 0, 7.5, 15, 20, 25, and 30 min and assayed for NOS activity as described.

Substrate Protection of Irreversible Inhibition by L-NMA. Incubation mixtures contained 2 μg of purified NOS, 700 μM DTT, 100 μM NADPH, 60 μM H_4B, 25 μM L-NMA, and 1 mM L-arginine or 25 μM L-NMA and 1 mM D-arginine in 15 mM Hepes (20% glycerol, v/v), pH 7.4, in a total volume of 320 μL. Incubations were assayed for NOS activity at 0, 5, 10, 15, 20, and 25 min.

Effect of SOD and Catalase on NOS Inactivation by L-NMA. Incubation mixtures contained 3 μg of NOS, 700 μM DTT, 100 μM L-NMA, 60 μM H_4B, and 150 units of SOD, or 100 units of catalase, both enzymes, or neither enzyme in 15 mM Hepes (20% glycerol, v/v), pH 7.4, in a total volume of 300 μL. Aliquots were removed at 0, 5, 10, 15, 20, and 25 min and assayed for NOS activity as described.

Effect of HCHO on NOS Activity. Incubation mixtures contained 3 μg of NOS, 700 μM DTT, 100 μM NADPH, 60 μM H_4B, and up to 1 mM HCHO in 15 mM Hepes (20% glycerol, v/v), pH 7.4, in a total volume of 300 μL. Aliquots were removed to 0, 5, 10, 15, 20, and 25 min and assayed for NOS activity as described.

Reversible Inhibition of NOS Activity by L-NMA. Purified NOS (1 μg) was combined with 100 μM NADPH, 70 μM DTT, 15 μM L-NMA, 6 μM H_4B, 4 μM oxyHb, and 12.5, 16.7, 25, 33, 50, or 100 μM L-arginine in 15 mM Hepes, pH 7.4, in a total volume of 500 μL and assayed for NOS activity as described.

RESULTS

Identification of Amino Acid Products from Excess^2 L-NMA and L-NHMA. As shown in Figure 3A, NOS generates amino acids from excess L-NMA (t_R = 22.7 min) which cochromatograph with L-NHA (t_R = 21.0 min), L-citrulline (t_R = 21.4 min), L-arginine (t_R = 21.8 min), and L-NHMA (t_R = 23.2 min). Incubations carried out with excess L-NHMA as the substrate were found to produce only one amino acid product which cochromatographed with L-citrulline (Figure 3B).

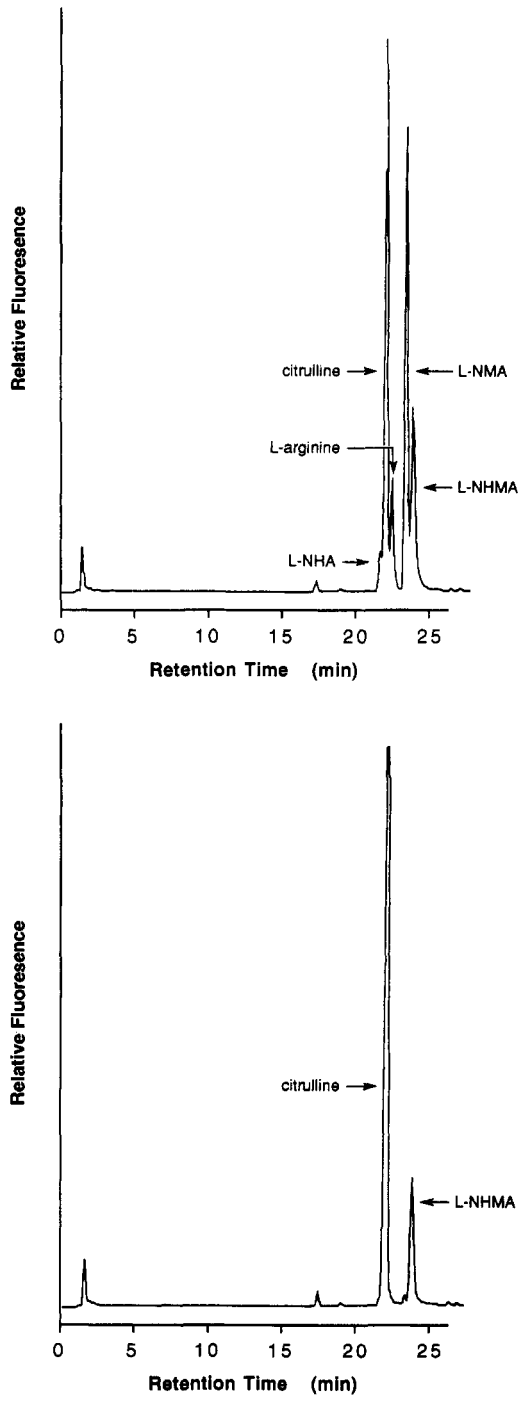


FIGURE 3: RP-HPLC chromatogram of amino acid reaction products formed by NOS from excess^2 L-NMA. Reactions which contained NOS, cofactors, and L-NMA were analyzed for amino acid content by precolumn derivatization with Fluoraldehyde reagent, followed by RP-HPLC and fluorescence detection. Peaks were identified as L-NHA (t_R = 21.0 min), citrulline (t_R = 21.4 min), L-arginine (t_R = 21.8 min), unreacted L-NMA (t_R = 22.7 min), and L-NHMA (t_R = 23.2 min) by co-injection with amino acid standards as described in Materials and Methods. The injection peak (t_R = 1.8 min) and a Fluoraldehyde peak (t_R = 17.5 min) are also seen. (B) RP-HPLC chromatogram of amino acid reaction product formed by NOS from L-NHMA. Peaks were identified as citrulline (t_R = 21.4 min) and unreacted L-NHMA (t_R = 22.7 min).

Identification of Amino Acid Products from Excess^2 L-[^{14}C]-NMA or L-[^{3}H]NMA. Analytical TLC of incubation mixtures containing excess L-[^{3}H]NMA were found to produce two

Table I: Stoichiometry of Citrulline to NO_x^- Formation from L-NMA[a]

expt	[citrulline][b] (μM)	[NO_x^-][c] (μM)	[citrulline]:[NO_x^-]
1	7.7 ± 0.4	7.3 ± 0.1	1.05
2	10.8 ± 0.1	10.7 ± 0.1	1.01
3	23.3 ± 0.1	23.8 ± 0.1	0.98

[a] All values are the average of duplicate measurements. [b] Citrulline was measured as [^{3}H]citrulline, as described in Materials and Methods. [c] $NO_x^- = NO_2^- + NO_3^-$. NO_x^- was determined by reduction of NO_3^- to NO_2^- and quantitation of NO_2^- with the Griess reagent as described in Materials and Methods.

major tritiated products which cochromatographed with L-citrulline and L-NHMA. Incubation mixtures containing excess L-[^{14}C]NMA were found to produce one major carbon-14-labeled product which cochromatographed with L-NHMA. Strong-cation-exchange HPLC with radiochemical detection (not shown) provided a more sensitive means of detection of radiolabeled amino acid products. From L-[^{3}H]NMA peaks of radioactivity corresponding to [^{3}H]citrulline, L-[^{3}H]NHA, L-[^{3}H]NHMA (t_R = 60 min), L-[^{3}H]NMA, and L-[^{3}H]-arginine could be assigned, although radiolabeled standards were not available for L-NHA and L-NHMA. From L-[^{14}C]NMA, peaks of radioactivity corresponding to [^{14}C]HCHO, L-[^{14}C]NHMA (t_R = 60 min), and L-[^{14}C]NMA were detected.

Amino Acid Analysis of Incubations with Limited[2] L-NMA, L-[^{3}H]NMA, or L-[^{14}C]NMA. Reactions carried out with limited L-NMA were analyzed by RP-HPLC. The only amino acid product detected cochromatographed with L-citrulline. Similar incubations carried out with limited L-[^{3}H]NMA or L-[^{14}C]NMA were analyzed by strong-cation-exchange HPLC with radiochemical detection. The only tritiated product detected from L-[^{3}H]NMA cochromatographed with [^{3}H]-citrulline. The only carbon-14-labeled product detected from L-[^{14}C]NMA eluted in the void volume and was identified as [^{14}C]HCHO (see below).

Identification of HCHO as the Demethylation Product from L-NMA. The identification of HCHO as the demethylation product was accomplished using two independent methods. The first utilized the Nash reagent for the spectrophotometric detection of HCHO as a 2,4-dihydrolutidine derivative (Werringloer, 1978). Approximately 1 equiv of HCHO was detected relative to [^{3}H]citrulline from L-[^{3}H]NMA or relative to NO_x^- from L-NHMA (not shown). The second method utilized [^{14}C]HCHO formed from L-[^{14}C]NMA. This was identified as its 2,4-dinitrophenylhydrazone derivative (Shriner et al., 1980) by utilizing RP-HPLC and concurrent UV and radiochemical detection (Banik & Silverman, 1990). Aliquots of sample were derivatized and analyzed by RP-HPLC as described in Materials and Methods. A large peak with a t_R = 5.6 min was evident by both UV and radiochemical detection (not shown). This peak cochromatographed with the authentic 2,4-dinitrophenylhydrazone of HCHO. RP-HPLC analysis of the reaction mixture without derivatization demonstrated only an injection peak (t_R = 1.8 min) by both UV and radiochemical detection.

Stoichiometry of Citrulline to NO_x^- Formation from L-NMA. The results from three representative experiments are shown in Table I. A 1:1 stoichiometry of citrulline to NO_x^- formation from L-[^{3}H]NMA was observed in all cases. Each group of samples was run with a no-substrate control, and each sample was analyzed separately for [^{3}H]citrulline and NO_x^- as described.

Stoichiometry of [^{14}C]HCHO to [^{3}H]Citrulline Formation from L-NMA. (i) Excess L-NMA. [^{14}C]HCHO and L-[^{3}H]-citrulline formation were measured simultaneously following incubation of NOS with cofactors and a substrate solution of ^{3}H- and ^{14}C-labeled L-NMA. As shown in Table II, the stoichiometry of [^{14}C]HCHO to [^{3}H]citrulline formed from excess ^{3}H- and ^{14}C-labeled L-NMA by NOS was 1.8:1.

Table II: Stoichiometry of HCHO to Citrulline Formation from Excess L-NMA[a]

expt	[HCHO][b] (μM)	[citrulline][b] (μM)	[HCHO]:[citrulline]
1	7.4 ± 0.1	4.2 ± 0.2	1.76
2	21.6 ± 1.5	11.6 ± 0.2	1.86
3	40.5 ± 0.4	24.7 ● 0.4	1.64

[a] All values are the average of duplicate measurements. Initial concentration of ^{14}C- and ^{3}H-labeled L-NMA was 250 μM. [b] HCHO was quantitated as [^{14}C]HCHO concurrently with [^{3}H]citrulline as described in Materials and Methods.

Table III: Stoichiometry of HCHO to Citrulline Formation from Limited[2] L-NMA[a]

expt	[HCHO][b] (μM)	[citrulline][b] (μM)	[HCHO]:[citrulline]
1[c]	10.6 ± 0.2	10.5 ± 0.2	1.01
2[d]	12.5 ± 0.1	12.4 ± 0.02	1.01

[a] All values are the average of duplicate measurements. [b] HCHO was quantitated as [^{14}C]HCHO concurrently with [^{3}H]citrulline as described in Materials and Methods. [c] Initial concentration of ^{14}C- and ^{3}H-labeled L-NMA was 10 μM. [d] Initial concentration of ^{14}C- and ^{3}H-labeled L-NMA was 12 μM.

(ii) Limited L-NMA. As shown in Table III, incubations containing NOS, cofactors, and limited ^{3}H- and ^{14}C-labeled L-NMA formed [^{14}C]HCHO and [^{3}H]citrulline in a stoichiometry of 1:1.

Stoichiometry of NADPH Consumption to [^{3}H]Citrulline Formation from L-NMA.[3] Plots of citrulline formed vs NADPH added were linear and intercepted the origin. The reciprocal of the slope of a line fit to this plot yielded to stoichiometry of NADPH consumption to [^{3}H]citrulline formation, which was 17.0 ± 0.3. In a control experiment, NOS which had been inactivated by preincubation with L-NMA was not found to oxidize NADPH at a significant rate relative to active enzyme (determined by following A_{340}). Thus, although L-NMA inactivated a portion of NOS during these reactions, only active NOS contributed to the observed stoichiometry of NADPH consumption.

Stoichiometry of H_2O_2 Formation to NADPH Oxidation for L-NMA or L-NHMA. The finding that the stoichiometry of NADPH oxidation to citrulline formation was 17:1 for L-NMA suggested that significant uncoupling of NADPH oxidation occurred. Plots of H_2O_2 formed vs NADPH added were linear and intercepted the origin. The slope of this plot yielded the stoichiometry of H_2O_2 formation to NADPH oxidation, which was 0.70 ± 0.02:1. Similar analysis using L-NHMA as substrate demonstrated a stoichiometry of 0.75 ± 0.01:1. Incubations carried out with L-arginine demonstrated low concentrations of H_2O_2 ($\leq$5 μM).

Initial Velocity of ·NO Generation from L-NMA and L-NHMA. When L-NMA or L-NHMA was used as substrate, the oxyHb assay for ·NO was not linear. This anomalous behavior was not attributable to the fact that L-NMA and

[3] The exact stoichiometries of NADPH oxidation to citrulline and ·NO formation for the substrates L-arginine and L-NHA are currently under investigation in our laboratory. However, each monooxygenase-like hydroxylation depicted in Scheme I would be expected to require one molecule of NADPH. The conversion of L-NHA to ·NO and citrulline is an NADPH-requiring process as well, with a stoichiometry of $\leq$1 molecule of NADPH.

L-NHMA are time-dependent inactivators of NOS activity. The addition of 100 units of catalase caused the assay to become linear for ≥3 min and increased (~2.0×) the measured initial rate with L-NMA or L-NHMA as substrate. The addition of 150 units of SOD also linearized the initial rate of the assay, but had a lesser effect (≤1.3×) on the magnitude of the increase in the initial rate than did catalase. Addition of 150 units of SOD and 100 units of catalase resulted in a larger increase in initial rate (~2.5×) than was produced by either enzyme alone. The measured initial rate of ·NO generation from L-arginine was increased to a much lesser degree when 150 units of SOD or 100 units of catalase or both (≤1.3×) were included in the oxyHb assay. In the presence of SOD and catalase the V_{max}(L-arginine)/(L-NMA) was equal to 20:1, and the V_{max}(L-arginine)/(L-NHMA) was equal to 13:1. The $K_{m\ app}$ values of L-NMA and L-NHMA were determined using the oxyHb assay modified with the addition of SOD and catalase as described. The $K_{m\ app}$ values of L-NMA and L-NHMA were found to be 3.1 ± 0.1 and 7.4 ± 1.3 μM, respectively. For comparison, the $K_{m\ app}$ of L-arginine was determined to be 7 μM in the presence of SOD and catalase.

Using the assay for [^{3}H]citrulline, NOS formed [^{3}H]-citrulline from L-[^{3}H]arginine or L-[^{3}H]NMA 1.1× faster in the presence of SOD and catalase than in their absence. This indicates that the effects of SOD and catalase in the oxyHb assay (described above) are attributable to their ability to scavenge H_2O_2 or O_2^- and not direct stimulation of NOS.

Enantiospecificity of NOS for L-Arginine and L-NMA. L- but not D-arginine and L- but not D-NMA generated detectable levels of ·NO in the oxyHb assay. Neither 500 μM D-arginine nor 500 μM D-NMA inhibited the rate of ·NO formation from 100 μM L-arginine.

NADPH Dependence of ·NO and Citrulline Formation from L-NMA and L-NHMA. In the absence of added NADPH, NOS did not generate detectable levels of ·NO from either L-NMA or L-NHMA as determined with the oxyHb assay. Incubations carried out in the absence of added NADPH demonstrated no amino acid other than substrate by RP-HPLC. Incubation mixtures which contained 100 μM NADPH demonstrated extensive turnover of substrate to ·NO or citrulline, as determined by the oxyHb assay or RP-HPLC, respectively.

Inhibition of NOS by L-NMA. Reversible inhibition of NOS by L-NMA was investigated using the oxyHb assay. A $K_{i\ app}$ equal to 13 μM was derived from a Lineweaver–Burk analysis of the data. L-NMA was found to inactivate NOS in a time- and concentration-dependent fashion, displaying pseudo-first-order kinetics of inactivation, $k_{inact} = 0.07\ min^{-1}$, and $K_I = 2.7$ μM. Preincubation mixtures with concentrations of D-NMA as high as 100 μM did not demonstrate rates of loss of NOS activity greater than controls which contained NOS and cofactors alone. Substrate protection from L-NMA inactivation was investigated with L- and D-arginine. Incubations which contained 25 μM L-NMA and 1 mM L-arginine lost activity at a rate equal to a control (which contained cofactors and NOS alone). Incubation mixtures which contained 25 μM L-NMA and 1 mM D-arginine were found to lose NOS activity at a rate essentially the same as with L-NMA alone.

Since ·NO and HCHO are formed from L-NMA by NOS, the effect of these molecules on NOS activity was investigated. Concentrations of HCHO as high as 1 mM resulted in rates of loss of NOS activity only slightly faster than control incubations with NOS and cofactors alone. Typical concentrations of HCHO generated from L-NMA in preincubation mixtures used for kinetic analysis were less than 20 μM. Preincubation mixtures which contained L-arginine (from which NOS forms ·NO 20 times faster than from L-NMA), NOS, and cofactors consistently lost the least amount of activity over time, and this was in fact the most stable preincubation mixture.

Effect of SOD and Catalase on NOS Inactivation by L-NMA. The finding (described above) that NOS processing of L-NMA is associated with H_2O_2 formation suggested that H_2O_2 or its potential precursor superoxide anion could contribute to the inactivation. However, preincubations carried out with 100 μM L-NMA in the presence or absence of SOD and catalase lost activity at the same rate (essentially, $k_{inact} = 0.07\ min^{-1}$). Preincubations carried out with 100 μM L-NMA with either SOD or catalase alone also lost activity at this rate. Preincubations carried out with NOS, cofactors, SOD, and catalase did not lose substantial activity over the duration of the preincubation.

DISCUSSION

The results described above show that L-NMA functions as a partially uncoupled, slow alternate substrate and mechanism-based inhibitor of purified macrophage NOS. The inactivation displays pseudo-first-order inactivation kinetics, saturability, and substrate protection. NOS formed ·NO, citrulline, HCHO, L-NHA, L-arginine, and L-NHMA by oxidation of the methyl-bearing guanidino nitrogen of L-NMA (Figure 3A). NOS formed ·NO, citrulline, and HCHO from L-NHMA (Figure 3B). NADPH oxidation was partially uncoupled from substrate oxygenation for both L-NMA and L-NHMA, resulting in the accumulation of H_2O_2. These results, taken together, suggest that L-NMA and L-NHMA undergo slow processing at the active site of NOS, which can culminate in ·NO and citrulline formation.

A proposal for L-NMA processing consistent with these results and the mechanism of NOS as a cytochrome P-450 is shown in Scheme I. Citrulline is thought to result from generation of either L-arginine or L-NHA at the active site following *N*-demethylation. *C*-hydroxylation of L-NMA would produce a carbinolamine. Subsequent collapse of this carbinolamine in the active site would generate L-arginine and HCHO. Release of the unstable carbinolamine (or L-arginine itself) from the active site would lead to the accumulation of L-arginine in solution, because the overwhelming excess of L-NMA would preclude further processing of the L-arginine. Of course, L-arginine generated at the active site can be processed to citrulline and ·NO. Alternatively, *N*-hydroxylation of L-NMA at the methyl-bearing guanidino nitrogen would generate L-NHMA. Release of L-NHMA into solution from the active site prior to further oxidation would likewise result in the accumulation of L-NHMA in solution. The formation of L-NHA is rationalized by further oxidation of L-NHMA at the active site to a nitrone. Hydrolysis of the unstable nitrone would generate HCHO and L-NHA. Release of this nitrone from the active site would result in the accumulation of L-NHA in solution. L-NHA formed and retained at the active site would be processed to citrulline and ·NO.

One key question is whether L-NMA processing occurs exclusively at the methyl-bearing guanidino nitrogen. The initial amino acid product from hydroxylation at the unsubstituted terminal guanidino nitrogen would be N'^G-hydroxy-N^G-methyl-L-arginine. Further oxidation of N'^G-hydroxy-N^G-methyl-L-arginine should generate ·NO and N^ω-methyl-

citrulline. N^{ω}-[^{14}C]Methylcitrulline would be expected to chromatograph similarly to citrulline, which is well resolved from other reaction products by both TLC and strong-cation-exchange HPLC. However, no evidence for the formation of this molecule was found (described above). Thus L-NMA, like L-NHA, appears to undergo oxidation exclusively at the functionalized terminal guanidino nitrogen. This may indicate that the L-arginine binding site of NOS cannot accommodate functionality at the terminal guanidino nitrogen *not* undergoing oxidation. This active site geometry would ensure that oxidation of only one terminal guanidino nitrogen occurred per catalytic cycle.

NOS converts 1 molecule of L-arginine to 1 molecule of citrulline and 1 molecule of ·NO (detected as NO_2^- and NO_3^-) (Tayeh & Marletta, 1989). As shown in Table I, quantification of [^{3}H]citrulline and NO_x^- formed from L-[^{3}H]NMA likewise yielded a 1:1 stoichiometry. Scheme I predicts that demethylation of L-NMA must precede formation of citrulline. The finding (described above) that significant concentrations of the demethylation products L-arginine and L-NHA accumulated in solution when L-NMA was in excess suggested that not all demethylation was linked to citrulline formation. Therefore, when L-NMA was present in excess, the expected stoichiometry of HCHO to citrulline was >1:1. As shown in Table II, the stoichiometry of [^{14}C]HCHO to [^{3}H]citrulline formation from excess L-NMA was found to be 1.8:1. When L-NMA is present in limited concentrations, Scheme I predicts a 1:1 stoichiometry of HCHO to citrulline, as all L-NMA present initially would be converted to citrulline, ·NO, and HCHO. As shown in Table III, this was indeed the case. Scheme I also predicts that HCHO, citrulline, and ·NO should be produced from L-NHMA in equimolar amounts, and as described above, each of these was detected.

The stoichiometry of NADPH oxidation to citrulline formation from L-NMA was greater than expected, 17:1. Inspection of Scheme I indicates that regardless of whether *N*- or *C*-hydroxylation initiates L-NMA processing, each pathway to the formation of citrulline would be expected to require about three molecules of NADPH.[3] In addition, each molecule of citrulline formed from excess L-NMA is associated with the formation of one molecule of L-NHMA (estimated from HPLC) and one molecule of HCHO not linked to citrulline formation (Table II). If the formation of each molecule of L-NHMA and L-arginine (the major demethylation product) from L-NMA required one NADPH, then a total of five molecules of NADPH would be expected to be oxidized per molecule of citrulline formed. Since the observed stoichiometry was 17 NADPH oxidized per citrulline formed, as much as 71% ([17 − 5]/17 × 100) of the NADPH oxidation could have been uncoupled from substrate oxygenation. The stoichiometry of H_2O_2 formed to NADPH oxidized with L-NMA as substrate was found to be 0.70:1. Thus, all the uncoupled NADPH oxidation appears to generate H_2O_2. The stoichiometry of H_2O_2 formation to NADPH oxidation was also determined with L-NHMA (not shown). As with L-NMA, the stoichiometry of H_2O_2 formation to NADPH oxidation was found to be 0.75:1. With L-arginine as the substrate, only low concentrations of H_2O_2 were detected (≤ 5 μM).

Thus, the consumption of 17 molecules of NADPH by NOS processing L-NMA is associated with the formation of 12 molecules of H_2O_2, about 2 molecules of HCHO, 1 molecule each of L-NHMA and citrulline, about 0.5 molecule of L-arginine, and 0.3 molecule of L-NHA. It should be noted that the inactivation of NOS by L-NMA is not slowed by inclusion of SOD and catalase in preincubation mixtures. Nor can the inactivation be replicated by omission of L-NMA and inclusion of 1 mM HCHO. It appears, then, that NOS inactivation is the result of chemistry originating at the active site and is not due to the diffusion of H_2O_2 or HCHO back into the active site following their release into solution. We are currently investigating other features of this inactivation, including turnover dependence, irreversibility, and the stoichiometry of radiolabeling.

Uncoupling of NADPH oxidation for P-450 enzymes is typically associated with H_2O_2 formation (Nordblum & Coon, 1977); however, the formation of H_2O has been inferred (Gorsky & Coon, 1984). NOS has recently been shown to be capable of forming H_2O_2 as well. Heinzel et al. (1992) recently described the Ca^{2+}/calmodulin-dependent generation of H_2O_2 by the constitutive bovine cerebellar NOS in the absence of L-arginine. Cytochrome P-450$_{BM-3}$ is the only P-450 other than NOS found in nature to contain both a P-450 reductase and a heme-binding oxygenase domain in the same polypeptide (Nahri & Fulco, 1986). Significantly, NADPH oxidation for P-450$_{BM-3}$ (Boddupalli et al., 1990) and NOS (R. A. Pufahl, J. M. Hevel, and M. A. Marletta, unpublished results) is tightly coupled when these enzymes are processing their normal substrates, long-chain fatty acids and L-arginine, respectively. Thus, the high level of uncoupling described herein for L-NMA does not appear to be a typical feature of the catalytically self-sufficient P-450s.

H_2O_2 formation may arise because of an interruption at the final step of oxygen activation: heterolytic oxygen–oxygen bond cleavage. This could result from interference in protonation of the enzyme-bound ferric peroxide caused by the steric bulk of the N^G-methyl group. An alternative explanation may be found upon consideration of the mechanism of NOS. The mechanism of NOS begins with a monooxygenase-like hydroxylation of L-arginine to form L-NHA. We have speculated that ·NO could be formed following nucleophilic attack at the guanidino carbon of L-NHA by an enzyme-bound ferric peroxide (Marletta, 1993), analogous to the third step in the mechanism of the P-450 enzyme aromatase. If NOS processed L-NMA and L-NHA similarly, the attack of an enzyme-bound ferric peroxide at the guanidino carbon of L-NMA might be less favorable than with L-NHA, due to either the electron-donating properties of the N^G-methyl group or steric interference. The ferric peroxide would then break down in one of two ways: simple diffusion of H_2O_2 from the active site or oxygen–oxygen bond cleavage. The latter would give rise to the perferryl species (Fe^V=O) thought to be the active oxidant in most P-450 chemistry (Guengerich & Macdonald, 1990). L-NHMA could likewise undergo the same type of slow processing again, leading to additional H_2O_2 formation, potential dissociation of L-NHMA without further oxidation, or a second *N*-hydroxylation that would form a nitrone. The findings that L-NHMA is processed nearly as slowly as L-NMA to ·NO and citrulline, that its processing results in a similar stoichiometry of H_2O_2 formation from NADPH, and that it is found released from the active site in relatively high amounts in the presence of excess L-NMA are consistent with this interpretation.

L-NMA has been widely used *in vitro* and *in vivo* as the prototypical "reversible" inhibitor of the inducible and the constitutive NOS (Moncada et al., 1991). L-NMA has been administered successfully to humans as an experimental treatment for septic shock unresponsive to conventional therapy (Petros et al., 1991). In this paper we present data which suggests that the fate of L-NMA at the hands of NOS is far

more complex than has been previously thought. Work presented herein would suggest that prolonged exposure to L-NMA *in vivo* may result in a sustained decrease in inducible NOS activity due to irreversible inactivation. However, the initial phase of L-NMA treatment would be expected to be accompanied by the formation of H_2O_2, ·NO, citrulline, and HCHO prior to enzyme inactivation.

The mechanism of inactivation of NOS by L-NMA is now under investigation in our laboratory. Among the mechanisms suggested by the P-450 literature, heme modification or apoprotein alkylation seem the most likely at this time (Oritz de Montellano, 1986). Scheme I suggests that a number of Michael acceptors could be formed from intermediates in L-NMA processing, including an imine derived from the carbinolamine and the nitrone derived from L-NHMA. In addition, the relatively high levels of H_2O_2 formed at the active site concomitant with L-NMA processing may lead to an infrequent irreversible oxidative event such as amino acid modification or heme destruction.

ACKNOWLEDGMENT

The authors wish to thank Joanie Hevel, Robert Pufahl, and Kim White for many helpful comments and suggestions. L-NHA and L-NHMA were provided by Robert Pufahl. We would like to thank Jason Johnson and Grace Sun for their expert assistance with cell culture. Recombinant murine interferon-γ was the generous gift of Genetech, Inc.

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