Hydrogen Peroxide-Supported Oxidation of N^G-Hydroxy-L-Arginine by Nitric Oxide Synthase†

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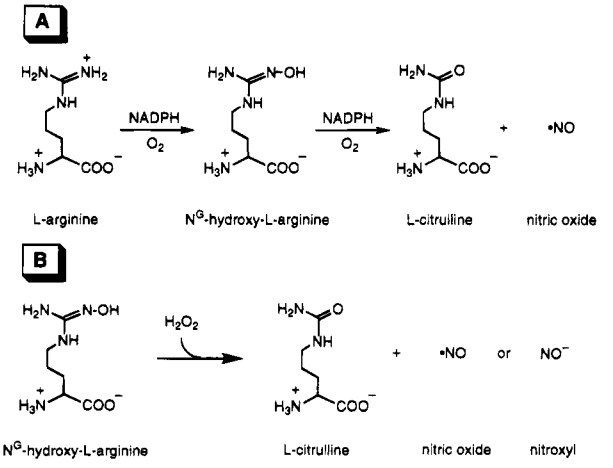
ABSTRACT: The ability of murine macrophage nitric oxide synthase (NOS) to utilize peroxides in place of O_2 and NADPH was investigated using hydrogen peroxide (H_2O_2), *tert*-butylhydroperoxide, and cumene hydroperoxide with both L-arginine and N^G-hydroxy-L-arginine (L-NHA) as substrates. Of the three peroxides examined, only H_2O_2 was able to support product formation using L-NHA as a substrate. No product formation was observed from L-arginine with any peroxide tested. Therefore, the L-NHA/H_2O_2 reaction was examined in greater detail. The products of the reaction were citrulline and nitrite/nitrate (NO_2^-/NO_3^-) with a stoichiometry of approximately 0.75:1 (citrulline to NO_2^-/NO_3^-). Product formation was greater in the presence of oxygen. Both the K_m and V_{max} of the reaction, determined under aerobic conditions, were affected by (6*R*)-tetrahydro-L-biopterin (H_4B). Chemiluminescence experiments failed to detect nitric oxide (•NO) as a reaction product. However, spectral experiments with L-NHA and H_2O_2 under anaerobic conditions demonstrated the appearance of a ferrous heme−•NO complex with a Soret peak at 440 nm and a broad single α/β peak at 578 nm, which is believed to arise from single electron transfer of a ferric−NO^- (nitroxyl) complex. Preliminary experiments detected nitrous oxide (N_2O) formation by gas chromatography under anaerobic conditions. Stable isotope labeling experiments with [^{18}O]H_2O_2 conclusively established incorporation of label exclusively into the ureido position of citrulline. Based on these results, a mechanism of oxidation of L-NHA by H_2O_2 is proposed.

Nitric oxide synthases (NOSs; EC 1.14.13.39),[1] unusual members of the cytochrome P450 family, catalyze the oxidation of L-arginine to nitric oxide (•NO),[2] and citrulline (Scheme 1A) at the expense of NADPH and O_2 (Marletta, 1993). Three isoforms of NOS are known to exist: a constitutive form found in the cerebellum (Bredt & Snyder, 1990; Schmidt et al., 1991), a constitutive form in endothelial cells (Pollock et al., 1991), and an endotoxin- and cytokine-inducible form, the best characterized example being that from mouse macrophages (Hevel et al., 1991; Stuehr et al., 1991a). The NOSs are cytosolic enzymes with the exception of the endothelial isoform, which is membrane-bound due, at least in part, to *N*-myristoylation (Michel et al., 1993; Busconi & Michel, 1993; Liu & Sessa, 1994). The major difference between the constitutive and inducible isoforms appears to be the method of regulation, although the isoforms are different gene products and show variations in molecular weight. Enzymatic activity of constitutively expressed NOS is strictly dependent on the association of Ca^{2+}/calmodulin to NOS, whereas the inducible isoform contains tightly bound nondissociating calmodulin (Cho et al., 1992; Stevens-Truss

Scheme 1. (A) NOS-Catalyzed Oxidation of L-Arginine and L-NHA to •NO and Citrulline and (B) Nitric Oxide or Nitroxyl Anion Formation from the Oxidation of L-NHA by H_2O_2



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[1] Abbreviations: •NO, nitric oxide; NO^-, nitroxyl anion; N_2O, nitrous oxide; NOS, nitric oxide synthase; NO_2^-, nitrite; NO_3^-, nitrate; NO_2^-/NO_3^-, total nitrite and nitrate; L-NHA, N^G-hydroxy-L-arginine; H_4B, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; H_2O_2 or HOOH, hydrogen peroxide; [^{18}O]H_2O_2 or $H_2{}^{18}O_2$, oxygen-18 labeled hydrogen peroxide; HPLC, high-performance liquid chromatography; GC-MS, gas chromatography−mass spectrometry; GC-ECD, gas chromatography−electron capture detection; EI, electron ionization; PCI, positive chemical ionization; BSTFA, *N,O*-bis(trimethylsilyl)trifluoracetamide; LPS, lipopolysaccharide; IFN-γ, interferon-gamma; PCA, protocatechuic acid; PCD, protocatechuate 3,4-dioxygenase; DTT, dithiothreitol; TCA, trichloroacetic acid.

[2] Registry No. (supplied by author): •NO, 10102-43-9; HNO, 14332-28-6; NO^-, 14967-78-3; L-NHA, 53054-07-2; H_2O_2, 7722-84-1; [^{18}O]H_2O_2, 29736-88-7; Piloty's acid, 599-71-3; citrulline, 372-75-8.

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and Marletta, unpublished observations) which presumably results in continuously elevated levels of activity. The isoforms, catalytically active as homodimers, contain one protoporphyrin IX heme iron (White & Marletta, 1992; McMillan et al., 1992; Stuehr & Ikeda-Saito, 1992) and one equivalent each of FAD and FMN (Hevel et al., 1991; Bredt et al., 1992). The stoichiometry of (6*R*)-tetrahydro-L-biopterin (H_4B) per NOS monomer appears to differ between isoforms. The macrophage isoform can contain stoichiometric levels of H_4B per subunit, although the H_4B content has been shown to vary between enzyme preparations (Hevel & Marletta, 1992). In contrast, brain NOS has been reported to contain one H_4B per dimer (Schmidt et al., 1992). By resonance Raman spectroscopy, the heme environment of brain NOS has been found to be similar to that observed for other cytochrome P450s (Wang et al., 1993).

N^G-Hydroxy-L-arginine (L-NHA), produced through the N-hydroxylation of one of the guanidino nitrogens of L-arginine (Scheme 1A) and normally released from NOS in only minute quantities during catalysis, is the only amino acid intermediate that has been identified in the reaction (Stuehr et al., 1991b; Pufahl et al., 1992; Klatt et al., 1993). NADPH and O_2 are required for both oxidations catalyzed by NOS, that is, from L-arginine to L-NHA and then from L-NHA to •NO and citrulline. The NADPH to •NO stoichiometry for the overall reaction with L-arginine as the substrate has been reported to be 1.5 while, starting with L-NHA, only 0.5 NADPH is required (Stuehr et al., 1991b). Evidence for participation of the heme in both oxidative steps has been shown through carbon monoxide inhibition studies (White & Marletta, 1992; Pufahl & Marletta, 1993) and optical difference binding studies of substrates (Pufahl & Marletta, 1993; McMillan & Masters, 1993).

Additional support for the participation of the heme in catalysis can be obtained from studies using organic hydroperoxides and hydrogen peroxide (H_2O_2) as oxygen donors in the P450 catalytic cycle. Organic hydroperoxides such as cumene hydroperoxide, or more rarely H_2O_2, are known to substitute for NADPH and O_2 in the catalytic cycle of cytochrome P450s in a pathway known as the peroxide shunt (Nordblom et al., 1976; White & Coon, 1980; Ortiz de Montellano, 1986). It is generally accepted that a high oxidation state iron−oxygen species, formally designated as either Fe(V)=O or $(Fe-O)^{3+}$, is formed from the reaction between hydroperoxides and ferric hemes, and this is the activated iron−oxygen species responsible for substrate oxidation. The same high oxidation state iron−oxygen species is presumably generated from NADPH and O_2, hence the terminology peroxide shunt. Therefore, this type of heme chemistry with hydroperoxides potentially could be used as models for the *N*-hydroxylation of L-arginine, as it is believed that this species may be responsible for the oxidation of L-arginine to L-NHA (White & Marletta, 1992). Similarly, iodosobenzene is known to generate an iron(V)−oxene species independent of both NADPH and O_2 (Gustafsson et al., 1979; Blake & Coon, 1989) and could also be used to probe the mechanism in a similar manner. In addition, the oxidation of L-NHA could also be examined with these systems. Given our current understanding of the oxidation of L-NHA to •NO and citrulline, the use of peroxides could potentially provide some key mechanistic details on this step in the reaction. On the basis of current knowledge, mechanistic speculation would predict the formation of nitroxyl (NO^-),[3] the one electron reduction product of •NO, from H_2O_2 and L-NHA (Scheme 1B). Interestingly, chemical model studies on *N*-hydroxyguanidine compounds and L-NHA itself suggest that either •NO or NO^- may be formed from these compounds by various oxidants (Fukuto et al., 1992, 1993). We report here our findings on the reaction of various organic hydroperoxides and iodosobenzene with NOS with the substrates L-arginine and L-NHA.

EXPERIMENTAL PROCEDURES

Materials and General Methods. H_4B was purchased from Dr. B. Schircks Laboratory (Jona, Switzerland) and was prepared in 100 mM Hepes (pH 7.5) containing 100 mM DTT. Oxyhemoglobin (human A_o, ferrous), catalase (bovine liver), Hepes, glycerol, DTT, NADPH, Dulbecco's modified Eagle's medium, LPS, penicillin and streptomycin were purchased from Sigma Chemical Company. Bovine serum albumin was obtained from Boehringer Mannheim. Macrophage RAW 264.7 cells were obtained from American Type Culture Collection (ATCC TIB 71). Calf serum was purchased from HyClone Laboratories and heat-inactivated at 56 °C for 30 min before use. DEAE Bio-Gel A, AG 50W-X8 cation exchange resin, disposable Poly-prep columns, Coomassie Blue R-250, and Bradford protein dye reagent were purchased from Bio-Rad. 2′,5′-ADP Sepharose 4B was purchased from Pharmacia-LKB Biotechnology Inc. Centrifugal filtration units (Ultrafree-MC, 10 000 molecular weight cut-off) were purchased from Millipore and were washed with water before using. Reacti-vials were obtained from either Pierce Chemical Co. (Rockford, IL) or Wheaton (Millville, NJ). N^G-Hydroxy-L-arginine was a gift from Abbott Laboratories (Abbott Park, IL), and elemental analysis yielded the molecular formula of NHA•2.2 HCl•0.1 pyridine (Wagenaar & Kerwin, 1993). NO_2^- and NO_3^- concentrations were determined as previously reported (Green et al., 1982). •NO gas (99%) was supplied by Matheson (Chicago, IL) and was further purified by passage through a saturated KOH solution. H_2O_2 (30% solution) was supplied by either Fluka (puriss) or Sigma. $H_2{}^{18}O_2$ was supplied by Icon (Summit, NJ) as a 2% solution with 90 atom % oxygen-18 enrichment. Iodosobenzene was obtained from Pfaltz and Bauer and used without further purification. Iodobenzene diacetate, cumene hydroperoxide (80%), and *tert*-butylhydroperoxide (70%) were supplied by Aldrich. *N*-Hydroxybenzenesulfonamide (puriss) was obtained from Fluka. BSTFA was purchased from Supelco, Inc. (Bellefonte, PA) and used without further purification. Acetonitrile was distilled from calcium hydride. Catalase activity was defined as given by the supplier; one unit decomposes 1 μmol of H_2O_2 per min at pH 7.0 at 25 °C while the H_2O_2 concentration falls from 10.3 to 9.2 mM. Protocatechuate 3,4-dioxygenase (PCD) and protocatechuic acid (PCA) were gifts from Dr. David Ballou (University of Michigan). PCD activity was measured in 100 mM Hepes (pH 7.5); 1 unit is defined as the amount of enzyme that will catalyze the formation of 1 μmol of product per min at 25 °C. All

[3] Nitroxyl (NO^-) or nitrosyl hydride is shown either in the deprotonated form or as HNO throughout this paper. Speculation on the exact identity of the protonated species, either HNO or NOH, exists in the literature. The difference in nomenclature is meant to represent either the singlet or triplet state of the molecule. However, no identification of the electronic state is made in this work.

distilled water was obtained from a Milli-Q water purification system (Millipore).

Purification of NOS. Cell culture procedures, induction of murine macrophage NOS activity, and the preparation of 100000*g* supernatant were carried out as described previously (Tayeh & Marletta, 1989) with the following modifications: 11−13 plates (150 × 25 mm) of confluent RAW 264.7 cells were activated for 14−20 h with 1 L of Dulbecco's modified Eagle's medium containing 10% heat-inactivated calf serum, 4 mM L-glutamine, penicillin (50 units/mL), streptomycin (50 μg/mL), *Escherichia coli* LPS (75 ng/mL), and recombinant murine IFN-γ (3.5 units/mL). Activated macrophages were harvested as described previously (Tayeh & Marletta, 1989). Purification of NOS was carried out as described previously (Hevel & Marletta, 1992) using 2′,5′-ADP Sepharose 4B and DEAE Bio-Gel A resins and a concentration step in an Amicon ultrafiltration system with a 50K cutoff ultrafiltration membrane (Filtron, Northborough, MA). The inclusion of 5 μM H_4B in all buffers during the purification and the concentration step resulted in NOS that contained nearly 1 pterin per subunit. NOS purified in this manner typically exhibited approximately a 25−30% (upper limit) rate enhancement on exogenously added H_4B as measured by the oxyhemoglobin assay. Protein was determined by the Bradford protein microassay using bovine serum albumin as a standard. NOS purified by this method was greater than 98% pure as judged by SDS-PAGE stained with Coomassie Blue R-250.

Preparation of Oxygen Donors. H_2O_2 solutions were prepared in 100 mM Hepes, pH 7.5; concentrations of H_2O_2 were determined spectrophotometrically at 240 nm in water using an extinction coefficient of 43.6 M^{-1} cm^{-1} (Beers & Sizer, 1952). Iodosobenzene, iodobenzene diacetate, and cumene hydroperoxide were prepared in methanol immediately before use. *tert*-Butylhydroperoxide was prepared fresh in water. Concentrations of cumene and *tert*-butylhydroperoxides were determined from data given by the supplier.

UV−Vis Absorbance Spectroscopy and Anaerobic Procedures. UV−vis spectroscopy was carried out with a Cary 3E spectrophotometer equipped with a Neslab RTE-110 circulating water bath. Experiments described here were carried out at 15.0 ± 0.1 °C. In some cases, enzyme solutions were made anaerobic by a combination of vacuum line and argon-purging techniques with a gas-train constructed in our laboratory. Oxygen concentrations could typically be reduced to 1−2 μM. Further anaerobiosis required PCA and PCD (see individual experiments for concentrations). Anaerobic cuvettes were 1 cm path length (for volumes of 400−500 μL), and septa were made of red rubber.

H_2O_2 Experiments with L-NHA

Product Identification. (A) The amino acid product was identified using three different techniques.

(i) Reverse-phase HPLC (RP-HPLC) of *o*-phthalaldehyde (OPA) derivatives with fluorescence detection was done as previously described (Olken & Marletta, 1993) except that tetrahydrofuran was omitted in the mobile phase. The retention time of citrulline under these conditions was approximately 21.5 min.

(ii) Strong-cation exchange chromatography was done with a Beckman model 6300 amino acid analyzer by Dr. William E. O'Brien at Baylor University, Medical School, Houston, TX. The retention time of citrulline was 35 min while that of L-NHA was 94 min.

(iii) Gas chromatography−mass spectrometry. Derivatization of the amino acid product(s) was done by using *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) followed by subsequent GC-MS analysis. Amino acid derivatizations were carried out in reacti-vials (1 mL) by adding acetonitrile (25−30 μL), pyridine (10 μL), and BSTFA (volume equal to that of the acetonitrile) to the reacti-vial containing the dried reaction mixture (see below). The vials were capped with silicone septa and heated to 100 °C and held there for a total of 1 h (includes heat-up time). Solutions were injected directly into the gas chromatograph after the vials cooled to room temperature. The GC-MS analyses were carried out on a Hewlett Packard model 5890 gas chromatograph interfaced with a Hewlett model 5989A mass spectrometer. For the analyses described here, the mass spectrometer was operated in either the electron ionization mode (EI) or the positive chemical ionization mode (PCI) with methane as the reagent gas. The column was fused-silica capillary; 12.5 m × 0.22 mm i.d. coated with HP-1 with a film thickness of 0.33 μm. Helium was the carrier gas with a head pressure of about 7 psi (50 kPa). The oven temperature was held at 75 °C for 0.5 min and then raised at 25 °C/min to 325 °C where it was held for an additional 0.5 min. Under these conditions, the retention time for the derivatized citrulline was approximately 6.3 min. Peak intensities in the PCI mass spectra were normalized to 100%.

(B) The nitrogen-containing product(s) were analyzed as follows:

(i) NO_2^- and NO_3^- (total and separately) by using the Griess reagent (Green et al., 1982).

(ii) •NO by chemiluminescence detection with a Sievers Model 270 •NO Chemiluminescence Detector (Boulder, CO) connected to a Hewlett Packard 3396A integrator. Samples (either aqueous or gaseous) were withdrawn from reacti-vials and injected directly into a glass manifold (designed by the manufacturer) containing water (1.5 mL). After sample injection, the system was pressurized by opening a stopcock from a nitrogen inlet for 5 s (to purge any •NO from solution into the head space), closing the inlet stopcock, and then opening an outlet stopcock connected directly to the detector. The head space of the apparatus was drawn into the detector by a vacuum pump (Edwards Model MO56LX2-A). After 5 s, the stopcock was closed and was ready again for another injection. However, due to the sensitivity of the instrument, several purge/vacuum cycles had to be performed between injections to remove residual •NO still present in the manifold. In addition, the manifold was rinsed with water after each assay to ensure that no signal was detected from a blank injection following cleaning.

(iii) N_2O formation by GC analysis with electron-capture detection (GC-ECD) as previously described (Kaspar & Tiedje, 1980; Parkin et al., 1984). Analysis was done with a Perkin-Elmer (model 910) GC with dual ^{63}Ni electron-capture detectors (standing current 1 mA; temperature, 300 °C) connected to a Hewlett Packard 3390A integrator. Gases were separated on a Porapak Q column (1.8 m × 0.32 cm o.d.) with a flow rate of 15 mL/min at 55 °C; the carrier gas was 95% argon and 5% methane. N_2O (retention time 3.32 min) was well separated from other gases using this system (nitrogen, 1.45 min; oxygen, 1.49 min; •NO, 1.54 min; carbon

dioxide, 2.23 min). Standard curves of N_2O were generated by injecting varying amounts of a 3.8 ppm N_2O standard (Matheson, Chicago, IL). Quantification of N_2O generated in enzymatic assays was done by injecting aliquots (400 μL) from the reacti-vial head space and then determining the total amount of N_2O in the gas and aqueous phases by using the Bunsen absorption coefficient and the following equation. Total N_2O amount (M) can be calculated from $M = C_g(V_g + \alpha V_l)$ where C_g equals the concentration of N_2O in the gas phase, V_g is the volume of the gas phase, V_l is the volume of the liquid phase, and α is the Bunsen absorption coefficient. For N_2O, α is 0.544 at 25 °C (Tiedje, 1994).

Time Course of NO_2^-/NO_3^- Formation with L-NHA/H_2O_2. The formation of NO_2^-/NO_3^- produced from L-NHA and H_2O_2 under aerobic conditions was observed with time at 37 °C. Assays (800 μL final volume) contained 100 mM Hepes (pH 7.5), L-NHA (500 μM), H_4B (40 μM), DTT (500 μM), NOS (11 μg), and H_2O_2 (25.4 mM). Some assays did not include H_4B or DTT. After initiation with H_2O_2, aliquots (75 μL) were removed at specified times and added to microfuge tubes containing TCA (120 mM final concentration). For comparison, an assay with NADPH (750 μM) and oxygen was done under identical conditions.

K_m Determination of H_2O_2. Assays (200 μL final volume) included 100 mM Hepes (pH 7.5), NOS (3.6−4.4 μg), L-NHA (200 μM), H_4B (40 μM), DTT (500 μM), and H_2O_2 (concentrations varied). All assays were initiated by the addition of H_2O_2 and were terminated after a 2 min period at 37 °C with either TCA (120 mM final concentration) or catalase (16 units; 10 μg of protein). In some instances, H_4B and DTT were not included. Oxygen was not excluded in these assays. The products, NO_2^- and NO_3^-, were measured by the Griess reagent. Nonenzymatically produced NO_2^-/NO_3^-, determined from identical assays without NOS, was subtracted at each H_2O_2 concentration. The amount of NO_2^-/NO_3^- produced nonenzymatically was typically less than 2% that observed in the presence of NOS.

Oxygen Dependence of L-NHA/H_2O_2 and L-NHA/O_2/NADPH Reactions. The dependence of the L-NHA/H_2O_2 reaction on molecular oxygen was examined under an argon atmosphere using protocatechuic acid (PCA)/protocatechuate 3,4-dioxygenase (PCD) as an oxygen scavenging system. To a borosilicate test tube (16 × 50 mm) fitted with a septum was added 100 mM Hepes, pH 7.5 (previously purged extensively with argon), L-NHA (267 μM), H_4B (20 μM), DTT (250 μM), NOS (15−20 μg), and PCA (267 μM). The assay mixture was blown gently with argon for 8 min on ice, PCD (0.40 units) was added by a gas-tight syringe, and then exposure to argon was continued for an additional 5 min. The reaction (final volume 750 μL) was initiated by the addition of anaerobic H_2O_2 (see Table 2 legend for concentrations) by a gas-tight syringe. The reactions were stopped after a 2 min period at 37 °C by the addition of TCA (120 mM final concentration) via a gas-tight syringe and subsequently analyzed for NO_2^-/NO_3^- formation. At the same time, an assay was done in the presence of oxygen (air-saturated solutions). In these cases, all of the components of the assay were identical to the oxygen-free assays except (i) PCD was not added and (ii) the H_2O_2 which was added did not contain PCD (but did contain PCA). Some assays were also done in the absence of added H_4B and DTT. The H_2O_2 stock solution was made anaerobic by thoroughly purging with argon followed by the addition of PCA (500 μM) and PCD (1.0 unit). As a control, identical assays containing NADPH (240 μM) were done (instead of H_2O_2) in the presence and absence of the oxygen scavenging system. Assays were initiated by the addition of NADPH.

Stoichiometry of Product Formation (Citrulline to NO_2^- and NO_3^-). The amount of citrulline and NO_2^-/NO_3^- formed from assays containing L-NHA and H_2O_2 was determined under both aerobic and anaerobic conditions. Levels of NO_2^-/NO_3^- were quantitated by the Griess reagent and citrulline by strong-cation exchange chromatography. Assays (aerobic, final volume of 500 μL; anaerobic, 750 μL) contained 100 mM Hepes (pH 7.5), NOS (7.5−22.5 μg), L-NHA (400 μM), H_4B (32 μM), DTT (400 μM), and H_2O_2 (varied between 16 and 40 mM). After the addition of H_2O_2, the reactions proceeded at 37 °C for 2−15 min followed by TCA termination (120 mM final concentration). In one aerobic experiment, H_4B and DTT were omitted. Complete assays with L-NHA and NADPH (500 μM) were also performed. Anaerobic assays containing PCA and PCD, which were done as described above, always included H_4B and DTT. All determinations of citrulline were corrected for recovery by adding a known amount of citrulline to an assay containing buffer, NOS, H_4B, DTT, and H_2O_2. Typically, this value was approximately 85%.

Spectral Studies of Nitrogen Product−NOS Complex Formation. Ferric NOS (1.4 μM), L-NHA (400 μM), H_4B (10 μM), DTT (500 μM), and PCA (250 μM) in 100 mM Hepes (pH 7.5) were added to an anaerobic cuvette fitted with a septum. This solution then underwent 10 cycles of purge/vacuum with a gas-train and was left under a positive pressure of argon. PCD (0.40 units) was added by a gas-tight syringe, and the cuvette was allowed to sit for 5 min. Anaerobic H_2O_2 (2 mM final concentration) was injected via a gas-tight syringe, and spectra were recorded every minute. Peroxide was made anaerobic as described earlier with 500 μM PCA and 0.60 units of PCD. Experiments with L-NHA under aerobic conditions and L-arginine under anaerobic conditions were also done (NOS 1.4 μM in both experiments). When the L-NHA experiment was carried out aerobically, conditions were identical to the anaerobic experiments except PCA/PCD was not used. When anaerobic experiments with L-arginine were done, everything was identical to the L-NHA experiments except L-arginine at a concentration of 400 μM was used.

•NO Chemiluminescence Experiments. •NO detection was performed as described above. Assays contained 100 mM Hepes (pH 7.5), NOS (8 μg), L-NHA (400 μM), H_4B (20 μM), DTT (250 μM), and either NADPH (250, 500, or 800 μM) or H_2O_2 (5, 10, 25, 30, or 50 mM) in a reacti-vial fitted with a silicone septum. In some instances, experiments were performed anaerobically using PCA (500 μM) and PCD (0.50 units); in those cases, H_2O_2 was made anaerobic as described earlier. All reactions were initiated with NADPH or H_2O_2 and proceeded at 37 °C. Aliquots (100 μL aqueous or head space) were removed periodically and analyzed for •NO. When studies with L-arginine were done, a concentration of 500 μM was used.

N_2O Experiments. Assays (300 μL) were carried out in reacti-vials (1 mL) fitted with a silicone septum and contained 100 mM Hepes (pH 7.5), glycerol (6−9%), NOS (18−27 μg), L-NHA (400 μM), H_4B (40 μM), DTT (500 μM), PCA (667 μM), PCD (0.60 units), and H_2O_2 (20 or 40 mM). Reactions were run under anaerobic conditions.

Buffer (previously purged with argon) was added to a reacti-vial containing L-NHA, H_4B, DTT, and PCA, and this mixture was bubbled with argon for 3 min. NOS (containing glycerol) was added to the reacti-vial, and the surface of the solution was blown gently with argon for an additional 5 min. Next, PCD was added via a gas-tight syringe, and reactions sat an additional 5 min to remove any remaining oxygen contaminant. H_2O_2, prepared anaerobically as described earlier, was then added to initiate the reactions. All assays proceeded at 25 °C for 15 min and were terminated with TCA (120 mM final concentration). Control assays lacked substrate or enzyme. Head spaces were sampled after sitting at room temperature for approximately 1 h; equilibration of gas from the liquid phase was necessary due to the high solubility of N_2O in aqueous solutions. Vigorous vortexing was also helpful.

Stable Isotope Studies Using Unlabeled and Oxygen-18 Labeled Hydrogen Peroxide. Assays (500 μL) contained 30 mM NH_4HCO_3 buffer (pH 7.8), L-NHA (400 μM), H_4B (35 μM), DTT (600 μM), NOS (20 μg), and 50 mM H_2O_2 (unlabeled or labeled). All reactions were initiated by the addition of H_2O_2, proceeded at 37 °C for 45 min, and were terminated by scavenging the remaining peroxide with catalase (20 units). Some experiments used NADPH (750 μM) in place of H_2O_2. Other experiments performed in the absence of L-NHA used authentic citrulline (300 μM). Exclusion of oxygen was not done. All components of the assays, including enzyme, were prepared in the volatile ammonium bicarbonate buffer. This was necessitated by the requirement that the GC-MS analysis be done under relatively salt-free conditions. All samples went through a work-up procedure to prepare the samples for GC-MS analysis. First, protein was removed by centrifugation of each sample through a Millipore 10 000 Da MW cut-off filter unit at 4 °C. Unreacted L-NHA was then removed from the assay mixtures by chromatography with Dowex AG50W-X8 cation exchange resin (sodium form; 0.6 mL of resin in a disposable Poly-prep column). The reaction product was eluted from the resin by washing with water (2 × 750 μL). Finally, samples were taken down to dryness with a SpeedVac concentrator, and the resulting residue was resuspended in water (500 μL) and dried again. This last procedure was repeated for a total of two times on each sample. Samples were then derivatized with BSTFA and analyzed by GC-MS (see above).

H_2O_2 Experiments with L-Arginine

Assays (200 μL final volume) contained 100 mM Hepes (pH 7.5), NOS (5 μg), L-arginine (200 μM), H_4B (40 μM), DTT (450 μM), and H_2O_2 (30, 50, 100, 150, or 200 mM). In some cases, H_4B and DTT were excluded. Oxygen was present in all assays. Assays were initiated by the addition of H_2O_2 and were terminated after 5 min at 37 °C with TCA (120 mM final concentration). NO_2^-/NO_3^- were measured by the Griess reaction as described earlier.

Other Oxygen Donors

Iodosobenzene and Iodobenzene Diacetate. Assays (200 μL) contained 100 mM Hepes (pH 7.5), NOS (1.9−3.5 μg), L-arginine (100 μM) or L-NHA (200 μM), and iodosobenzene or iodobenzene diacetate. H_4B or NADPH was not added. For assays containing L-arginine, iodosobenzene concentrations tested were 50 μM, 100 μM, 250 μM, 500 μM, or 1.0 mM. Assays with L-NHA used 50 μM, 100 μM, 250 μM, 1.0 mM, or 1.25 mM. In addition, L-arginine and L-NHA assays using iodobenzene diacetate were at 50 and 100 μM. All assays were initiated with the oxygen donor, proceeded at 37 °C for 5−10 min, and then were stopped by the addition of TCA (120 mM final concentration). Total NO_2^- and NO_3^- levels were then determined by the Griess reaction as well as analysis of amino acid products by RP-HPLC of OPA derivatives.

Cumene and tert-Butyl Hydroperoxides. Assays (200 μL) contained 100 mM Hepes (pH 7.5), NOS (4.0−6.0 μg), L-arginine (100 μM) or L-NHA (200 μM), and cumene or *tert*-butyl hydroperoxide. With L-arginine, cumene hydroperoxide concentrations were 100 μM, 500 μM, 1.5 mM, 10 mM, or 20 mM; *tert*-butyl hydroperoxide concentrations that were tested were either low (100 or 500 μM) or high (10, 20, 30, 40, or 50 mM). In assays containing L-NHA, low concentrations (250 μM, 500 μM, 1 mM, or 1.5 mM) as well as high concentrations (10 or 20 mM) of cumene hydroperoxide were examined. Assays with *tert*-butyl hydroperoxide used either with low concentrations (100 or 500 μM) or high concentrations (10, 20, 30, 40, or 50 mM). NADPH was not present in any assays and H_4B was omitted in most cases. However, H_4B (40 μM) and DTT (400 μM) were present in all assays using 10, 20, 30, 40, or 50 mM hydroperoxide. Reactions proceeded for at least 10 min at 37 °C, were terminated with either TCA (120 mM final concentration) or by freezing at −80 °C, and were then analyzed for NO_2^- and NO_3^-. Some assays were analyzed for amino acid products by RP-HPLC as described above.

RESULTS

Oxygen Donors as Substrates for NOS. Three peroxides, H_2O_2, *tert*-butyl hydroperoxide, and cumene hydroperoxide, were examined with macrophage NOS to evaluate whether or not the peroxide shunt pathway was operative. Both L-arginine and L-NHA were examined as substrates. Of the three peroxides, only H_2O_2 was able to support product formation and only when L-NHA was used as the substrate. Products were not observed from assays with L-arginine and any peroxide tested. Potential products from L-arginine oxidation might include L-NHA, citrulline, and/or NO_2^-/NO_3^-. Although examination of amino acid products by RP-HPLC with fluorescence detection of OPA derivatives is hampered by the relatively poor resolution of L-arginine and L-NHA, no evidence was obtained to support the formation of L-NHA. This observation was further substantiated by the fact that NO_2^-/NO_3^- was not formed from assays with L-arginine and peroxide, as it was found that H_2O_2 was quite able to mediate the oxidation of L-NHA to NO_2^-/NO_3^- (see below). Initial experiments with L-arginine containing H_2O_2 concentrations up to 30 mM were negative (a concentration which will support the oxidation of L-NHA); therefore, higher concentrations of H_2O_2 were utilized. However, there were negligible levels of NO_2^-/NO_3^- above background at concentrations as high as 200 mM.

Additionally, neither iodosobenzene nor iodobenzene diacetate was able to support product formation from L-arginine. Studies with L-NHA were complicated due to the fact that iodosobenzene reacted nonenzymatically with L-NHA to produce NO_2^-/NO_3^- stoichiometric with the

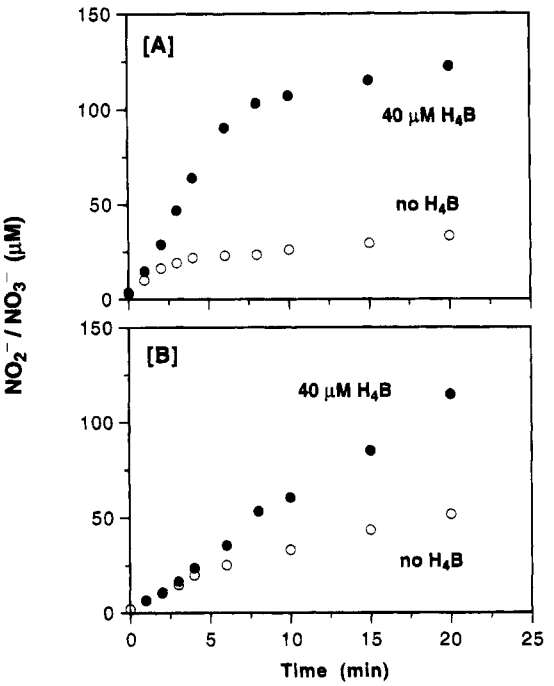


FIGURE 1: Time courses of product formation from L-NHA under aerobic conditions with either (A) H_2O_2 or (B) NADPH as substrates and the effect of H_4B on product formation. In each experiment, aliquots (75 μL) were removed from the original assay at the specified times and added to tubes containing TCA (120 mM final concentration). The original assays (800 μL each) contained 100 mM Hepes (pH 7.5), L-NHA (500 μM), and NOS (11 μg). Assays in panel A contained H_2O_2 (25.4 mM), and those in panel B contained NADPH (750 μM). When H_4B was present, the concentration was 40 μM and those assays also contained 500 μM DTT. Closed circles represent assays done in the presence of H_4B and DTT while open circles represent assays done without H_4B or DTT.

iodosobenzene consumed. However, enzymatic assays did not produce NO_2^-/NO_3^- above nonenzymatic levels. This reaction was not pursued further.

General Characteristics of the L-NHA/H_2O_2 Reaction. Initially NO_2^-/NO_3^- were identified as the stable nitrogen-derived products of H_2O_2-mediated oxidation of L-NHA. Subsequently, time course studies were done to determine the optimal time for measurement of enzyme activity. It is well known in the cytochrome P450 literature that high concentrations of hydroperoxides cause varying degrees of time-dependent heme destruction (Nordblom et al., 1976; Coon et al., 1982), and this was also a concern here. It was observed that the H_2O_2 supported reaction was partially dependent on H_4B. This observation has also been made for the NADPH/O_2 reaction with both L-arginine and L-NHA as substrates (Stuehr et al., 1991b; Pufahl et al., 1992; Klatt et al., 1993). The time course of NO_2^-/NO_3^- formation under aerobic conditions from the H_2O_2 reaction is shown in Figure 1A. Figure 1B shows a time course for an otherwise identical assay containing NADPH. Several differences between the two are apparent. First, it is obvious that the H_2O_2 reaction deviates from linearity before the NADPH supported reaction. In the presence of H_4B, the reaction is linear for at least 6 min while in the absence of H_4B, the reaction is linear for 2 min. This differs dramatically from the NADPH reaction, where in the presence of H_4B product formation is linear up to the end of the assay (20 min) and in the absence of reduced pterin the reaction tails off slowly after 5 min. Second, a greater H_4B dependence of product formation appears to exist with the peroxide reaction. Lastly, the initial rate of the H_2O_2 reaction (with pterin present) is greater than that formed in the NADPH reaction; however, product formation eventually ceases, and the amount of product is eventually surpassed in the NADPH reaction during longer assay times.

Table 1: Kinetic Parameters of the L-NHA/H_2O_2 Reaction[a]

	K_m (mM)	V_{max} (μM total NO_2^- and NO_3^-/μg NOS/2 min)
+H_4B ($n = 3$)	30.5 ± 3.8	29.4 ± 2.9
−H_4B ($n = 4$)	18.8 ± 4.4	8.2 ± 1.5

[a] The assays contained 100 mM Hepes (pH 7.5), NOS (3.6−4.4 μg), L-NHA (200 μM), and H_2O_2 (varied). Oxygen was not excluded. H_4B (40 μM) and DTT (500 μM) were included in some assays. All assays were done at 37 °C for 2 min followed by termination with TCA (120 mM final concentration). See Experimental Procedures for complete experimental details.

To determine the kinetic parameters of the H_2O_2 reaction, assays were stopped after a 2 min period at 37 °C followed by NO_2^-/NO_3^- quantitation. Background subtraction of nonenzymatically produced NO_2^- and NO_3^- at each peroxide concentration was done (product formation was generally less than 2% of that formed with inclusion of enzyme). In the presence of H_4B (40 μM) and DTT (500 μM), the $K_{m,app}$ of H_2O_2 was 30.5 ± 3.8 mM ($n = 3$), and the $V_{max,app}$ was 29.4 ± 2.9 μM total NO_2^- and NO_3^-/μg of NOS/2 min. Under experimental conditions similar to those used in the assays (100 mM Hepes, pH 7.5, 40 μM H_4B, 500 μM DTT, 20 mM H_2O_2, ±100 μM L-NHA), greater that 90% of the H_4B remained after 2 min. In the absence of reduced pterin and DTT, the $K_{m,app}$ decreased to 18.8 ± 4.4 mM ($n = 4$) while the $V_{max,app}$ decreased by 3.6-fold to 8.2 ± 1.5 μM total NO_2^- and NO_3^-/μg of NOS/2 min (Table 1). Both K_m and V_{max} are reported as apparent kinetic constants as L-NHA concentrations were not varied. The L-NHA concentration used in these studies was 200 μM, which is well above the reported K_m's of 6.6 (Stuehr et al., 1991b) and 28 μM for macrophage NOS (Pufahl et al., 1992).

The differences in K_m and V_{max} were not the result of DTT; assays containing DTT without H_4B gave the same amount of product as assays without DTT or H_4B (data not shown). In addition, the reaction displayed saturation kinetics at H_4B concentrations above 10 μM (data not shown). The reaction was enzyme-catalyzed; NO_2^-/NO_3^- and citrulline were not formed in the absence of either NOS or H_2O_2 (data not shown). Product formation was linear with enzyme concentration (data not shown) and neither ferric nor ferrous iron (ferric chloride or ferrous ammonium sulfate) showed any activity (data not shown).

The amino acid product of the reaction was shown by several chromatographic methods to be citrulline. No evidence was suggestive of the formation of any other amino acid product. Chromatographic identification of citrulline was by both RP-HPLC of OPA derivatives and strong-cation exchange chromatography and agreed with the retention times of identically treated citrulline standards. Unequivocal evidence was obtained by GC-MS of TMS-derivatives of the amino acid isolated from the reaction mixture. The amino acid product was partially purified from assay mixtures by removing excess H_2O_2, NOS, and unreacted L-NHA. Assays were carried out in a volatile ammonium bicarbonate buffer due to the interference of salt with GC-

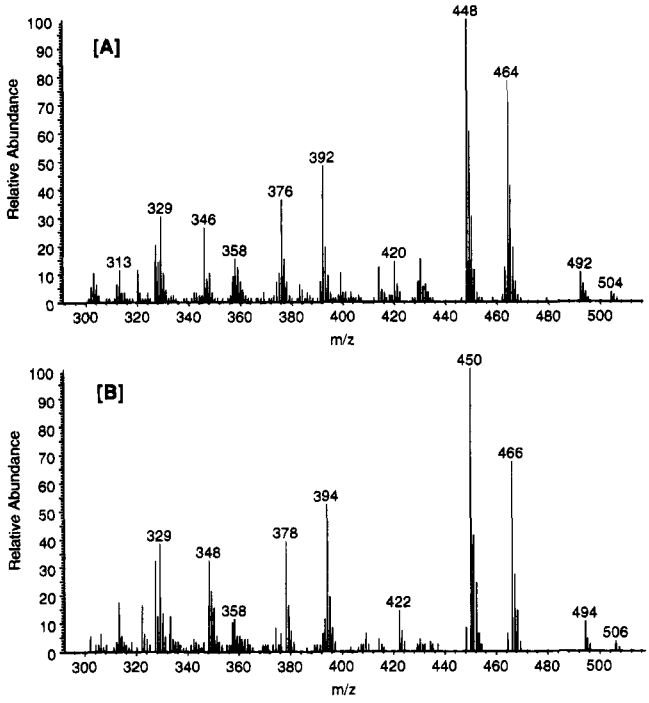


FIGURE 2: (A) PCI mass spectrum of BSTFA-derivatized citrulline obtained from the L-NHA/H_2O_2 reaction. (B) PCI mass spectrum of BSTFA-derivatized citrulline obtained from the L-NHA/[^{18}O]-H_2O_2 reaction.

MS analysis. Samples were subjected to GC-MS analysis after BSTFA derivatization. Citrulline was identified by both PCI and EI mass spectrometric techniques. The PCI mass spectrum (Figure 2A) with methane clearly showed a $(TMS)_4$-citrulline derivative with molecular ions at *m/z* 464 (MH^+) and *m/z* 448 as well as the expected higher adducts at *m/z* 492 (M+29) and *m/z* 504 (M+41). Additional fragments were observed at *m/z* 420, 392, 376, 346, and 329. The EI mass spectrum (not shown) demonstrated a parent ion of *m/z* 463 (M^+) with lower fragments at *m/z* 448, 346, 313, 232, 188, 142, 117, and 102. The molecular ion at *m/z* 142 gave the greatest intensity. Both spectra were in excellent agreement with derivatized authentic citrulline as well as enzymatically produced citrulline from assays containing NADPH and O_2 (not shown).

Two heme ligands were examined as inhibitors of the L-NHA/H_2O_2 reaction. Carbon monoxide (CO), a ligand for ferrous heme, was demonstrated previously to act as an inhibitor of the NADPH catalyzed oxidation of both L-arginine and L-NHA (Pufahl & Marletta, 1993). In that study, CO (80:20 mixture of CO/oxygen) inhibited the reaction approximately 57% and 33% with L-arginine and L-NHA as substrates, respectively. It has been observed that with peroxide-supported reactions of P450s, however, CO does not inhibit the reaction since reduced ferrous heme is not formed during the peroxide shunt cycle (Nordblom et al., 1976). In this study, CO was a very poor inhibitor of the peroxide mediated oxidation of L-NHA. The inhibition was variable; in some instances ($n = 3$), no inhibition was observed while in others ($n = 1$), up to 10% inhibition was found (data not shown). Therefore, little inhibition, if any, was afforded by CO.

Cyanide has also been characterized as an inhibitor of cytochrome P450s. Unlike CO, cyanide binds to ferric hemes. Reports on cyanide inhibition of NOS have been few; however, Mayer and co-workers have reported an IC_{50} of 4.2 mM for potassium cyanide for citrulline formation with neuronal NOS (Klatt et al., 1993). Preliminary experiments with potassium cyanide (10 mM) with macrophage NOS indicate 61% inhibition of L-arginine/NADPH activity (measured as NO_2^-/NO_3^-), 37% inhibition of L-NHA/NADPH activity and 55% inhibition of L-NHA/H_2O_2 activity. Thus, cyanide is an effective inhibitor of each reaction.

Table 2: Oxygen Dependence of the L-NHA/H_2O_2 and L-NHA/NADPH Reactions[a]

	nitrite/nitrate (μM)	
experiment	air	argon
1	22.7	5.5
2	22.4	5.7
3	23.6	5.1
4	26.1	6.8
5	24.2	6.4
6	37.2	11.2
7	14.1	6.2
8	12.1	6.0
9	14.6	9.8
10	16.2	1.5
11	12.4	1.3

[a] The oxygen dependencies of the L-NHA/H_2O_2 and L-NHA/NADPH reactions are shown above. Complete experimental details are given in Experimental Procedures. Experiments 1−6 and 10 were done in the presence of H_4B (20 μM) and DTT (250 μM) while 7−9 and 11 were done in the absence. H_2O_2 concentrations were 14.3 mM (experiments 1−3), 14.8 mM (experiments 4−5 and 7−8), and 27.1 mM (experiments 6 and 9). NADPH (240 μM) was present in experiments 10 and 11. In addition, all experiments were 2 min reactions.

Superoxide dismutase (SOD) had little inhibitory effect on the L-NHA/H_2O_2 reaction. At either 40 or 150 units of SOD, the reaction was inhibited approximately 10%. However, 10% inhibition was also observed when SOD was included in assays containing L-NHA and NADPH. Thus, superoxide anion does not appear to play an important role in either reaction.

Oxygen Requirement. The experiments described above were all carried out in the presence of air. Experiments designed to examine the oxygen requirement of the L-NHA/H_2O_2 reaction revealed that the amount of NO_2^-/NO_3^- produced under anaerobic conditions was substantially less than that formed in air (Table 2). Anaerobiosis was obtained by using argon-purged buffers and then removing the last oxygen contamination by a PCA/PCD oxygen scavenging system. H_2O_2 inhibited PCD slightly at the concentrations used (data not shown); therefore, larger amounts of PCD were used. In the presence of H_4B (experiments 1−6), the amount of product formed under aerobic conditions was approximately 3−5 times greater than that under anaerobic conditions. This result (4.2 times less) did not change when saturating levels of H_2O_2 were used in the assay (data not shown). This ratio was diminished in the absence of reduced pterin (experiments 7−9).

Experiments were also done with L-NHA in the presence of NADPH (experiments 10 and 11). The requirement for oxygen is clearly demonstrated in this case. Little product is formed from L-NHA under anaerobic conditions.

Stoichiometry of Products. The stoichiometry of product formation (citrulline and NO_2^-/NO_3^-) produced under various assay conditions was investigated. Under aerobic conditions and in the presence of H_4B, the stoichiometry of

citrulline to NO_2^-/NO_3^- formed was determined to be 0.74 ± 0.12 (n = 8). The stoichiometry did not change as the assay length was altered (from 2 to 15 min). In addition, the stoichiometry (0.85) was affected slightly in the absence of H_4B. Under anaerobic conditions, however, a product stoichiometry of greater than one was observed (data not shown). In three experiments, the stoichiometry varied from 1.5 to 2.4, definitely suggestive of the possibility of an unaccounted product if one assumes the products (amino acid and nitrogen-derived) are formed in equimolar amounts. In aerobic or anaerobic experiments there was no evidence for the formation of another amino acid product.

Assays done in the presence of NADPH and O_2, where it is known that the stoichiometry of citrulline to NO_2^-/NO_3^- formation is approximately unity (Pufahl et al., 1992), exhibited a stoichiometry of 1.02 ± 0.19 (n = 2) in this set of experiments (not shown).

Nitrogen-Derived Product from L-NHA Oxidation. The identification of the primary nitrogen-derived product from the oxidation of L-NHA by H_2O_2 is of great mechanistic value. Several candidates for this product include •NO, NO^-, or perhaps N_2O (nitrous oxide), which is the product of nitroxyl dimerization and dehydration (Bonner & Hughes, 1988). Direct identification of •NO can be accomplished by chemiluminescence detection, a method widely used because of its excellent sensitivity. In this method, •NO reacts with ozone to produce an electronically excited state of nitrogen dioxide. Relaxation to the ground state occurs upon photon release, which is detected with a photomultiplier tube.

Aerobic or anaerobic L-NHA/H_2O_2 assays were examined for •NO production both in the aqueous and gas phases. However, the results did not indicate the generation of •NO under either condition. Experiments under similar conditions with the NADPH/O_2 reaction with both L-arginine and L-NHA easily detected •NO (data not shown). The lack of detection in the peroxide reaction is probably not due to the production of insufficient quantities of •NO, as the peroxide reaction produces larger amounts of NO_2^-/NO_3^- at earlier times than the NADPH reaction (assuming that most of the NO_2^-/NO_3^- is derived from •NO decomposition). Another compelling piece of evidence is that the half-life of •NO should be greatly increased under decreased oxygen tensions, making it easier to observe any •NO. However, •NO analysis is complicated by the presence of high concentrations of H_2O_2 necessary for the reaction to proceed. These concentrations of H_2O_2 oxidize •NO under the assay conditions, although not all •NO is oxidized at earlier time points of the assay (data not shown). Thus, it should be possible to observe some •NO formation if indeed •NO is produced.

Another method for the detection of either •NO or NO^- is by electronic absorption spectroscopy through the formation of transiently stable heme complexes. Both ferric– and ferrous–nitrosyl hemes of cytochrome P450s have been well characterized by UV–vis spectroscopy (O'Keeffe et al., 1978; White & Coon, 1982). Initially, several difficulties in the formation of these complexes of NOS were encountered; however, these problems were solved when experiments were done under decreased H_2O_2 concentrations and lower temperatures. High concentrations of H_2O_2 at 37 °C resulted in rapid heme destruction (data not shown), thereby making it impossible to study product binding to NOS. However, under anaerobic conditions at 15 °C and 2 mM

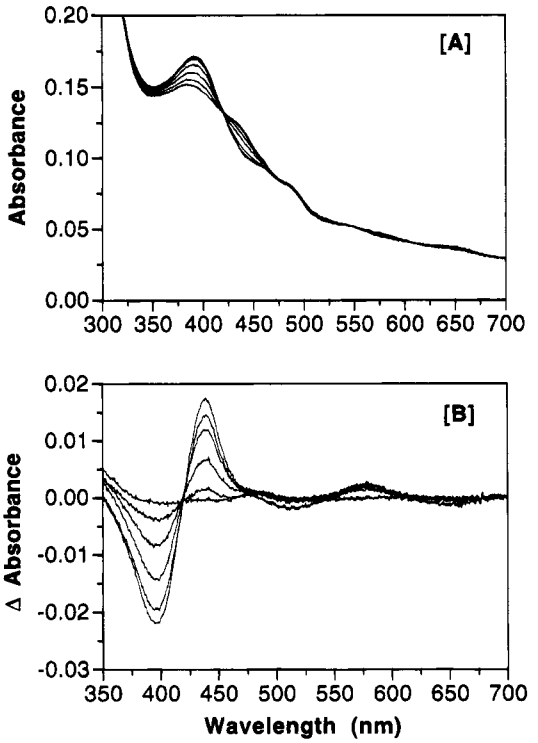


FIGURE 3: (A) UV–vis absorbance spectroscopy of transient complex formed from NOS, L-NHA (400 μM), and H_2O_2 (2 mM) under anaerobic conditions at 15 °C. Spectra were recorded every minute; for clarity, only spectra at time equals 0, 2, 4, 6, 8, 10, 12, and 14 min are shown. See Experimental Procedures for complete details. (B) Selected difference spectra of NOS–product complex from the experiment described in panel A. Spectra were calculated by subtracting the original substrate-bound high-spin ferric NOS from subsequent spectra containing H_2O_2.

H_2O_2, a transient NOS-product complex (Figure 3A) was observed. Difference spectra (Figure 3B) clearly show the formation of a species with an increase of the Soret at 440 nm, a decrease at 394 nm, and a broad single α/β peak at 578 nm. The complex formed within 2 min and started to decay after an additional few minutes; by 15 min, ferric enzyme was regenerated.

Interestingly, this complex did not form when only H_2O_2 was added (not shown); L-NHA had to be present before H_2O_2 addition. The complex did not form under aerobic conditions (not shown); anaerobiosis was a requirement. Finally, spectral studies under identical conditions to the L-NHA experiments (in which the transient complex was observed) were carried out with L-arginine. Complex formation was not observed; in fact, a small decrease in absorbance of the heme Soret was observed over time (data not shown).

The identity of this NOS–product complex agrees with the literature spectroscopic values of ferrous heme–nitrosyl complexes of both soluble and microsomal P450s (O'Keeffe et al., 1978; White & Coon, 1982). Nitrosyl complexes of cytochrome P450s generally have a Soret absorbance at approximately 435–440 nm and additional absorbances in the α/β region between 540 and 585 nm. The distinguishing feature between ferric– and ferrous–•NO complexes are not in the Soret absorbance but rather in the α/β region. In all cases examined thus far, ferric–•NO complexes exhibit two distinct peaks in this region whereas ferrous–•NO complexes have one. Ferric–•NO complexes have absorbances at approximately 540 and 570 nm while the ferrous counterparts

have one peak at approximately 585 nm. Both the ferric–and ferrous–nitrosyl complexes of NOS have been formed in our laboratory and agree with the spectroscopic data of other P450s. By difference spectroscopy, the ferric nitrosyl complex of NOS has a Soret at 444 nm and α/β peaks at 552 and 588 nm, while the ferrous nitrosyl NOS has a Soret at 442 nm and a broad single α/β peak at 581 nm (Hurshman and Marletta, unpublished observations).

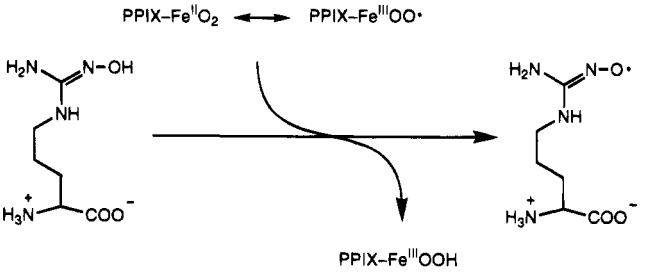
Stable Isotope Labeling Studies with $H_2{}^{18}O_2$. Stable isotope studies aimed at understanding the mechanism of oxidation of L-NHA were done using $[^{18}O]H_2O_2$ under aerobic ($^{16}O_2$) conditions. Incorporation of label into citrulline was then determined by GC-MS analysis after derivatization of citrulline with BSTFA. The $(TMS)_4$-$[^{18}O]$-citrulline was detected by both PCI- and EI-MS methods. PCI mass spectrometry showed several fragments shifted by 2 mass units in comparison to unlabeled H_2O_2 samples (Figure 2B). Shifted molecular ions were observed at *m/z* 450, 466 (MH^+), 494 (M+29), and 506 (M+41) as well as the lower ions at *m/z* 422, 394, 378, and 348. In addition, the EI mass spectrum also clearly identified the incorporation of label into citrulline. Two mass unit shifted ions were found at *m/z* 465 (M^+), 450, 348, 234, and 190; other ions were found at *m/z* 313, 142, 117, and 102 (mass spectrum not shown). Both PCI- and EI-MS showed total incorporation of oxygen-18 label into citrulline (>90%).

DISCUSSION

Although much progress has been made in the general characterization of NOS isoforms and in certain aspects of •NO formation, the chemical steps in the reaction remain, for the most part, unknown. In fact, the only clearly established step characterized thus far is the initial hydroxylation of L-arginine to L-NHA as shown in Scheme 1A. The most detailed mechanistic proposal has been based on several important observations (Marletta, 1993). The sequence homology to NADPH cytochrome P450 reductase first reported for the neuronal NOS isoform isolated from rat cerebellum (Bredt et al., 1991), with the subsequent demonstration of the presence of a bound FAD and FMN (Hevel et al., 1991; Stuehr et al., 1991a), and the associated diaphorase activity (Hope et al., 1991) all pointed toward an internal redox shuttling role for this apparent domain of the enzyme. Subsequent studies then showed that all NOS isoforms contained a cytochrome P450 type heme (White & Marletta, 1992; McMillan et al., 1992; Stuehr & Ikeda-Saito, 1992) which provided both a sink for reducing equivalents shuttled by the flavoprotein reductase domain and a cofactor capable of carrying out at least some of the NOS chemistry. The inhibition of the reaction by CO with either L-arginine or L-NHA as substrates further implicated the heme in catalysis (White & Marletta, 1992; Pufahl & Marletta, 1993). Although H_4B normally functions as a cosubstrate in hydroxylation reactions, no evidence supports this type of a function in the NOS reaction.

The initial hydroxylation of L-arginine to L-NHA is speculated to occur via typical P450 chemistry involving the perferryl oxidation state of the heme iron, $(FeO)^{3+}$, which results from cleavage of the O–O bond. Further oxidation of L-NHA has to take into account the observations that the citrulline which is produced contains oxygen derived from molecular oxygen (Kwon et al., 1990) and that one-electron chemistry must occur at some point in the reaction in order to generate •NO. This has led to the proposal that, with L-NHA oxidation, chemistry takes place before O–O bond scission and involves an iron peroxide nucleophile as has been proposed in the reaction catalyzed by aromatase (Akhtar et al., 1993, 1994). The ability of non-iron-bound peroxide to function as a nucleophile in reactions with aldehydes has been reported (Cole & Robinson, 1991). Furthermore, certain liver P450 isoenzymes will oxidize cyclohexane carboxaldehyde using H_2O_2 to produce cyclohexene and formate, products analogous to those generated by aromatase (Vaz et al., 1991).

Scheme 2. Proposed Oxidation of L-NHA by the Ferrous Oxy Heme Complex of NOS[a]



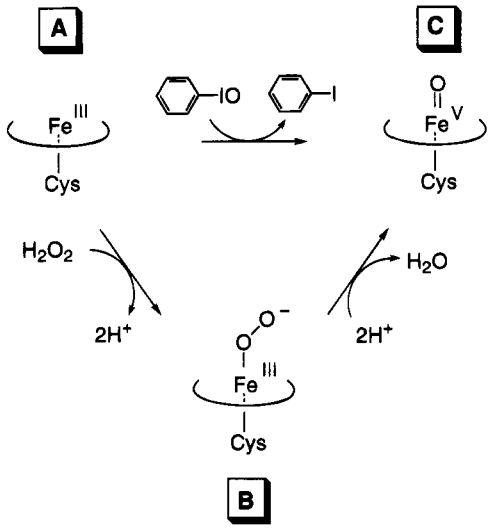
[a] A key step in the proposed mechanism of L-NHA conversion to citrulline and •NO involves a one-electron oxidation of L-NHA to form an amino acid radical intermediate that ultimately reacts further to generate •NO (Marletta, 1993).

The current mechanistic proposal of •NO formation is based on all the results obtained to date as well as the literature precedent for P450s to carry out chemistry using a ferric peroxide as a nucleophile. The first step of the reaction is envisioned to involve the hydroxylation of L-arginine by the $(FeO)^{3+}$ complex of the heme to produce L-NHA. As illustrated in Scheme 2, the next step in the reaction is proposed to involve oxidation of L-NHA by the $Fe^{II}O_2$ heme complex. This one-electron-oxidized L-NHA would then generate •NO after reaction with the ferric peroxide and collapse of the resulting tetrahedral intermediate (Marletta, 1993). On the basis of the redox potential of P450 $Fe^{II}O_2$ heme complexes versus that of L-NHA, a modification to this oxidation has been proposed involving hydrogen atom abstraction from L-NHA by the ferric superoxide complex (Korth et al., 1994).

This mechanism then has three distinguishing features: (i) N-hydroxylation by an $(FeO)^{3+}$ equivalent, (ii) one-electron oxidation of the substrate, and (iii) chemistry before O–O bond scission involving an iron peroxide nucleophilic reaction. The studies reported here were designed to test two of these features by generating the $(FeO)^{3+}$ and the Fe^{3+}–OOH species directly in the absence of NADPH. On the basis of the knowledge concerning the mechanism, it was expected that agents such as iodosobenzene which directly generate $(FeO)^{3+}$ would hydroxylate L-arginine to L-NHA. On the other hand, peroxide-supported chemistry was also expected to carry out the N-hydroxylation of L-arginine to L-NHA and then the further oxidation of L-NHA to citrulline and nitroxyl (NO^-). NO^- is the expected nitrogen-derived product, and not •NO, since the peroxide-supported reaction would not generate the heme ferrous–oxy complex, the species speculated to carry out the one-electron oxidation of L-NHA when NADPH and O_2 are the substrates.

As stated above, the N-hydroxylation of L-arginine by NOS is not supported by iodosobenzene, and, although

Scheme 3. Alternative Oxygen Donors in P450-Catalyzed Reactions[a]



[a] In typical P450 reactions, iodosobenzene has been shown to carry out reactions associated with the formation of the perferryl oxidation state [$(FeO)^{3+}$] shown as C in the scheme. This oxidation state can also be formed from H_2O_2 via intermediate B after heterolytic cleavage. Intermediate B has also been implicated in chemistry catalyzed by aromatase as well as in the NOS reaction reported here.

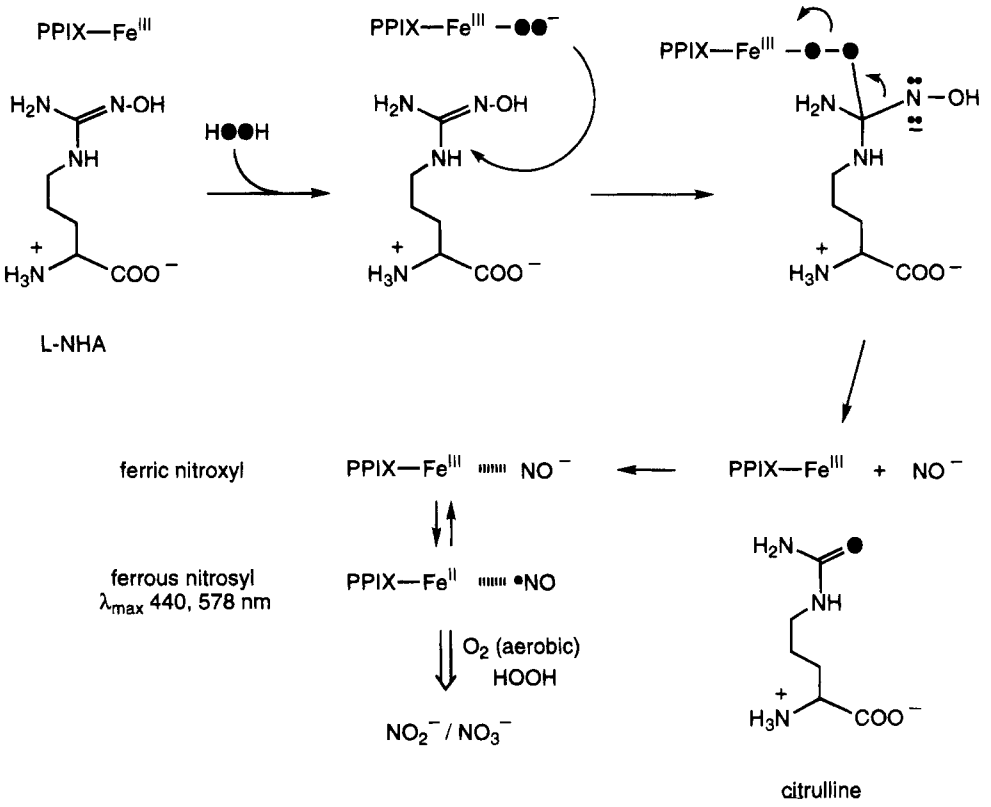
complicated by the fact that L-NHA reacts nonenzymatically with iodosobenzene, iodosobenzene-mediated oxidation of L-NHA by NOS apparently does not occur. Iodosobenzene can serve as a source of active oxygen for many P450 reactions; however, the failure of iodosobenzene to support the oxidative chemistry carried out by NOS may be due to several reasons. Perhaps the active-site of NOS, which is expected to be considerably more hydrophilic in nature than typical P450s, may not be able to accommodate this hydrophobic compound. On the other hand, it could be that the $(FeO)^{3+}$ species forms (Scheme 3C) but does not hydroxylate L-arginine, suggesting that this step in the reaction may not be carried out by the heme. The substrate specificity of NOS is quite narrow and a suitable compound to examine if indeed the $(FeO)^{3+}$ complex forms with NOS is not available. Interestingly, L-arginine is not a substrate in the reaction with H_2O_2 either. The reaction of H_2O_2 with NOS should lead to the same $(FeO)^{3+}$ intermediate that would be generated from iodosobenzene; this is illustrated as A → B → C in Scheme 3. It is possible that O−O bond scission in NOS does not occur. However, if this bond cleavage does take place, it may be irrelevant to the chemistry involved in the N-hydroxylation of L-arginine, if the heme is not responsible for this step in the reaction. This conclusion is consistent with the results obtained with iodosobenzene. Alkyl peroxides, which are typically better in the peroxide shunt than H_2O_2 in P450-catalyzed reactions, were not effective as oxygen donors in the NOS reactions. The active site of NOS is expected to be considerably more hydrophilic compared to typical P450s, and it may be difficult for hydrophobic alkyl peroxides to gain access to the heme. On the other hand, our results more directly support ferric peroxide as a nucleophile (Scheme 3B), which would not be possible with an alkyl substituted peroxide.

The peroxide-supported oxidation of L-NHA does appear to yield citrulline and NO^- as products as predicted. Formation of citrulline has been unambiguously demonstrated by GC-MS methods, and, as expected, the ureido oxygen atom of citrulline is derived exclusively from peroxide, supporting the hypothesis that a ferric-peroxide nucleophile is involved in the reaction. Characterization of the nitrogen-derived product has been indirect. Formation of NO^- would be expected to lead to N_2O via dimerization (Bonner & Hughes, 1988), and although some N_2O was indeed formed under certain conditions, the amounts were less than expected. GC-ECD analysis of the head space from anaerobic reaction mixtures consistently revealed that approximately 15−25 μM N_2O was formed. Control assays did not contain N_2O in any appreciable amount. The majority of the nitrogen-derived products were still NO_2^- and NO_3^-. Since these anions are known to be the solution decomposition products of •NO, it is possible that they were derived from •NO. We were unable to detect •NO formation by chemiluminescence detection, a sensitive and specific method to measure •NO. However, some •NO is oxidized by H_2O_2 under these assay conditions. Control assays with authentic •NO (concentrations similar to that generated in enzymatic assays containing L-NHA, NADPH, and O_2) in 100 mM Hepes (pH 7.5) containing 30 mM H_2O_2 resulted in the oxidation of some •NO, especially at later time periods (>5 min) as determined by •NO chemiluminescence experiments. In contrast, •NO was easily detected with this method under normal conditions of enzymatic turnover using NADPH and O_2. Furthermore, previous stoichiometry studies using NADPH and O_2 showed that L-NHA loss was equal to the citrulline and •NO (measured as NO_2^-/NO_3^-) formed (Pufahl et al., 1992).

The results from the experiments presented here can be integrated to give a mechanistic proposal which is consistent with these experimental findings (Scheme 4). Under aerobic conditions, the products of the reaction (citrulline and NO_2^-/NO_3^-) are formed in approximately equimolar amounts. Under anaerobic conditions, the same products are found although, based on the determined stoichiometry, an additional nitrogenous product may be formed. In agreement with this scenario, low levels of N_2O were measured. The nitrogen-derived product appears to be NO^-, which concurs with the results of the anaerobic UV−vis spectroscopy experiments as well as the lack of •NO detection in aerobic or anaerobic assays. The NO^- that is produced can form an anaerobically stable ferrous−nitrosyl complex which is catalytically inactive (i.e., limited turnover under anaerobic conditions) but decomposes under aerobic conditions to NO_2^- and NO_3^-. Oxidation of NOS-bound NO^- and the direct oxidation by H_2O_2 and O_2 in solution accounts for our inability to detect high amounts of NO^- (or N_2O). Indeed, model studies with *N*-hydroxybenzenesulfonamide (NHBS or Piloty's acid), a known precursor compound of NO^- (Bonner & Ko, 1992), in 75 mM sodium borate (pH 9.0) suggest that NO^- does yield NO_2^-/NO_3^- as products (data not shown). Both NO_2^- and NO_3^- were formed from the aerobic decomposition of NHBS whether H_2O_2 was present (30 mM) or not. Under anaerobic conditions, however, NO_2^-/NO_3^- formation was only observed from NHBS decomposition in the presence of H_2O_2.

Overall the results reported here provide support for the involvement of H_2O_2 in the NOS reaction in a manner analogous to that observed for aromatase and related P450 demethylases. CO inhibition of the reaction with either L-arginine or L-NHA as substrates is strongly suggestive of

Scheme 4. Proposed Mechanism of H_2O_2-Supported Oxidation of l-NHA by NOS[a]



[a] H_2O_2 is represented in this scheme as HOOH and oxygen-18 labeled atoms as ●. Nucleophilic attack by a ferric peroxide on the guanidino group of L-NHA yields a tetrahedral intermediate which collapses to yield citrulline and NO^-. NO^- complexes with ferric NOS which can undergo single electron transfer to form a ferrous−nitrosyl complex. This complex, observed at 440 and 578 nm, is proposed to react with HOOH to produce NO_2^-/NO_3^- under anaerobic conditions. In addition, some N_2O is also produced. Under aerobic conditions, the formation of NO_2^-/NO_3^- is proposed to occur by reactions involving O_2 and HOOH.

the direct participation of the heme in catalysis (Pufahl & Marletta, 1993) and led to the proposed mechanism (Marletta, 1993). Another possible scenario involves the attack of the $(FeO)^{3+}$ complex on the isolated double bond of the guanidine moiety of L-arginine. The subsequently generated oxaziridine would then be expected to open up to L-NHA. Another oxidative step involving this type of chemistry coupled with one-electron chemistry would be expected to generate •NO and citrulline. Since the oxygen atom in citrulline is known to be derived from O_2 (Kwon et al., 1990), this second step would require an unusual opening of the oxaziridine ring, perhaps on the one-electron-oxidized *N*-hydroxyoxaziridine intermediate. The question as to why L-arginine does not serve as a substrate regardless of which oxidant is used is intriguing. These results suggest that the initial hydroxylation of L-arginine may be carried out by a non-heme-dependent functional group. The role of the reduced pterin in the NOS reaction has not been determined and, until definitively ruled out, remains a viable option in this initial step of the reaction. Further examination of additional single oxygen donors with NOS will undoubtedly clarify the chemistry of the N-hydroxylation of L-arginine.

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