Formation of N^{δ}-Cyanoornithine from N^{G}-Hydroxy-L-arginine and Hydrogen Peroxide by Neuronal Nitric Oxide Synthase: Implications for Mechanism†

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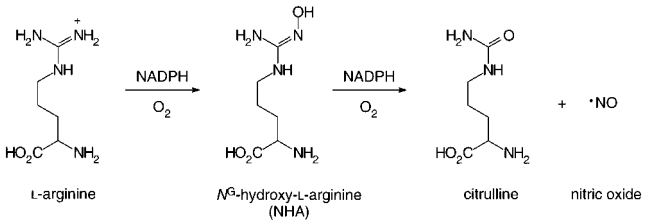
ABSTRACT: Neuronal nitric oxide synthase (nNOS) catalyzes the oxidation of N^{G}-hydroxy-L-arginine (NHA) by hydrogen peroxide. The amino acid products were characterized by high-performance liquid chromatography/mass spectrometry of the *o*-phthalaldehyde/2-mercaptoethanol derivatives and identified as citrulline and N^{δ}-cyanoornithine (CN-orn). The assignment of CN-orn was confirmed by independent chemical synthesis and comparison of the properties of the enzyme-derived product with those of synthetic CN-orn. The inorganic products detected in the enzymatic reaction were NO_2^- and NO_3^-, presumably from oxidation of NO^-. The reaction of H_2O_2 and NHA with nNOS was at least 10-fold slower than the reaction of NADPH, O_2, and NHA ($V_{max,app} = 49 \pm 2$ nmol min^{-1} mg^{-1} for the reactions with 10 μM added H_4B). The reaction exhibited saturation kinetics with respect to hydrogen peroxide [$K_{m,app}(H_2O_2) = 10 \pm 1$ mM for the reactions with 10 μM added H_4B]. No H_2O_2-dependent reaction was observed with L-arginine as the amino acid substrate. The different products for the NADPH- and H_2O_2-dependent transformations of NHA are of mechanistic significance in the NOS reaction.

Nitric oxide synthase (NOS,[1] EC 1.14.13.39) catalyzes the five-electron oxidation of arginine to citrulline and nitric oxide (*1, 2*). This NADPH- and O_2-dependent reaction (Scheme 1) has been shown to proceed via an intermediate, N^{G}-hydroxyarginine (NHA) (*3, 4*). Nitric oxide participates in important physiological processes, such as vasodilation and neuronal signaling. Despite intensive study, the mechanism of •NO formation remains poorly understood (*2, 5*). Three isoforms of NOS are known: one inducible form, the best characterized of which is purified from murine macrophages, and two constitutive forms, endothelial and neuronal (*5*). All three isoforms require calmodulin for activity. The constitutive forms bind calmodulin reversibly in response to the intracellular calcium concentration (*6, 7*), while the inducible isoform binds calmodulin nearly irreversibly and independent of calcium concentration (*1, 8*). In addition to calmodulin, each NOS subunit binds stoichiometric amounts of two flavins (FAD and FMN) (*9*−*12*), protoporphyrin IX-type heme (*12*−*15*), and tetrahydrobiopterin (H_4B) (*11, 12, 16*). NOS is composed of two functional domains: the reductase domain, where the flavins are bound, and the heme domain, where the oxidation of the substrate occurs. Reducing equivalents from NADPH are transferred via the flavins to the heme domain.

Scheme 1: Reaction Catalyzed by NOS



The heme domain binds heme and is also thought to bind H_4B. The role of reduced biopterin is unknown, but H_4B is strictly required for catalysis. The heme produces a characteristic cytochrome P450 spectrum upon reduction in the presence of CO, consistent with a cysteine thiolate ligand to the heme. This thiolate ligation suggested that NOS might exhibit P450 chemistry, which is best characterized for $P450_{cam}$ (CYP101). Indeed, the mechanism proposed for other P450 enzyme systems has been useful as a model for NOS chemistry. The first step of arginine oxidation, the *N*-hydroxylation of arginine to NHA, is a two-electron oxidation, the mechanism of which is unclear: some experimental data suggest the participation of the heme, while other data implicate an alternative cofactor, such as H_4B. P450-type hemes are known to catalyze related reactions. For instance, *N*-hydroxylation of tyrosine has been described

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[1] Abbreviations: nNOS, neuronal nitric oxide synthase; NHA, N^{G}-hydroxy-L-arginine; H_4B, *(6R)*-5,6,7,8-tetrahydro-L-biopterin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; CN-orn, N^{δ}-cyanoornithine; CaM, calmodulin; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenebis-(oxyethylenenitrilo)tetraacetic acid; HPLC, high-performance liquid chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; *o*-PA, *o*-phthalaldehyde; NDA, 2,3-naphthalenedicarboxaldehyde; TLC, thin-layer chromatography; FT-IR, Fourier transform infrared; NaOAc, sodium acetate; CcP, cytochrome *c* peroxidase; HRP, horseradish peroxidase; ClPO, chloroperoxidase; P450, cytochrome P450.

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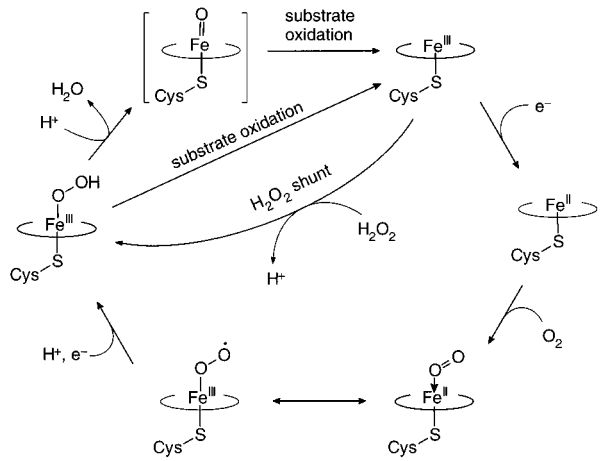
Scheme 2: Modified P450 Reaction Cycle[2]



for cytochrome $P450_{Tyr}$ (CYP79) (*17*). Hydroxylations are also catalyzed by reduced pteridines (*18*). The second step of arginine oxidation, the three-electron oxidation of NHA to citrulline and •NO, was proposed to be a heme-dependent process for the inducible isoform of NOS (*19*). Taken together, the following observations strongly implicate the heme in catalysis: NHA perturbs the spin state of the heme (*19*); CO inhibits catalytic turnover, implicating a reduced iron intermediate (*19*); and hydrogen peroxide substitutes for NADPH and O_2 (*20*), a functional substitution that is expected for heme-dependent oxidations based on the catalytic cycle shown in Scheme 2[2] (*21*, *22*).

We undertook a similar investigation of the H_2O_2-dependent reactions of the neuronal isoform. No reaction is observed when arginine is the amino acid substrate. When NHA is the amino acid substrate, an additional amino acid product, N^{δ}-cyanoornithine (CN-orn), is formed. The observation of this product during H_2O_2-dependent turnover, but not during NADPH-dependent turnover, has important mechanistic implications for both the H_2O_2-dependent and NADPH-dependent reactions.

EXPERIMENTAL PROCEDURES

Materials and General Methods. Spodoptera frugiperda (Sf9) cells, derived from an ATCC culture (CRL-1711) and adapted to Sf-900 II SFM, a serum-free medium, were obtained from Gibco-BRL. They were maintained in Sf-900 II SFM (Gibco-BRL), supplemented with 0.1% antibiotic solution (Sigma, A9909). H_4B was purchased from Dr. B. Schircks Laboratory (Jona, Switzerland) and prepared in 50 mM Hepes (pH 7.4) containing 100 mM DTT. Affinity resins, 2′,5′-ADP−Sepharose 4B and CaM−Sepharose 4B, were from Pharmacia-LKB Biotechnology, Inc. Bradford protein dye reagent was from Bio-Rad. Other reagents were from Aldrich and Sigma.

Expression and Purification of nNOS. Sf9 cells were infected with wild-type recombinant baculovirus (*23*). A hemin/BSA solution was made by dissolving 10 μmol of hemin [chloroprotoporphyrin IX iron(III)] and 110 mg of Na_2HPO_4 in 5 mL of 0.1 M NaOH. This solution was added to a solution of 100 μmol of BSA in 95 mL of H_2O. The pH was adjusted to ∼7. The resultant solution, 100 μM hemin and 1 mM BSA, was filtered through a 0.45 μm filter, stored at −80 °C, refiltered, and added to cells 12 h postinfection to a final hemin concentration of 4 μM. Infected cells were harvested after 60−70 h, centrifuged at 100000*g*, and stored at −80 °C, as described (*23*).

The purification of nNOS was modified from Richards et al. (1996). Supernatant was assayed by the oxidation of oxyhemoglobin (*24*), where 1 unit is defined as 1 nmol of •NO/min), and 1000−2000 units were loaded onto a 1.5 g 2′,5′-ADP Sepharose 4B column, which had been equilibrated with buffer A (50 mM Hepes, 0.5 mM arginine, 100 mM NaCl, 10% glycerol, and 8 μM H_4B, at pH 7.4) without H_4B. The column was rinsed with 10 mL of buffer A containing 0.1 mM EDTA and 0.1 mM EGTA and washed with 20 mL of buffer A containing 0.1 mM EDTA, 0.1 mM EGTA, and an additional 200 mM NaCl. The column was rinsed with 10 mL of buffer B (50 mM Hepes, 0.5 mM arginine, 100 mM NaCl, and 8 μM H_4B, at pH 7.4), and nNOS was eluted with 20 mL of buffer C (50 mM Hepes, 100 mM NaCl, 2 mM $CaCl_2$, and 8 μM H_4B, at pH 7.4) containing 5 mM NADPH directly onto a 4-mL CaM affinity column (2 mL of CaM−Sepharose 4B and 2 mL of Sepharose 4B) equilibrated with 50 mM Hepes, 100 mM NaCl, and 2 mM $CaCl_2$ at pH 7.4. The CaM column was washed with 15 mL of buffer C. nNOS was eluted with 10 mL of buffer C containing 10% glycerol and 5 mM EGTA and concentrated once to approximately 1 mg/mL in an Amicon filter unit using a 100 kDa MW cutoff membrane. Purified nNOS was stored at −80 °C with a total of 20% glycerol and 0−30 μM additional H_4B. Purity, assessed by SDS−PAGE and staining with Coomassie Blue R-250, was greater than 95%. Specific activity, measured by the oxidation of oxyhemoglobin, was between 400 and 800 nmol min^{-1} mg^{-1}. Protein concentrations were determined by the Bradford microassay using BSA as a standard and 160 kDa as the subunit mass of nNOS. All concentrations are reported as concentrations of nNOS subunit.

Quantitation of Amino Acids. Amino acids were derivatized by two different methods and separated by reverse-phase HPLC, using an HP 1090 Series II instrument with a diode array detector and a Nova-Pak C_{18} column (150 mm × 3.9 mm, 4 μm particle size, Waters Associates Inc.), equipped with a C_{18} guard column (Alltech Associates, Inc., Vydac, 7.5 × 4.6 mm). *o*-Phthalaldehyde/2-mercaptoethanol derivatives were made with 5 μL of a solution of 6 mM *o*-phthalaldehyde (*o*-PA) and 86 mM 2-mercaptoethanol in 1.0 M potassium borate buffer (pH 10.4) and 10 μL of sample. This reaction was mixed in the injector loop of the autosampler for 10 cycles (total elapsed time 3.2 min). Alternatively, amino acids were derivatized with 2,3-naphthalenedicarboxaldehyde (NDA) and sodium cyanide (*25*). In this case, 2 μL of 50 mM NaCN in 1.0 M potassium borate (pH 9.5), 1 μL of 10 mM NDA in methanol, and 7−10 μL of sample were mixed in the injection loop for at least 15 min (*26*). In both cases, samples were automatically applied to the column, which was maintained at 40 °C and equilibrated with 5 mM ammonium acetate (pH 6.0) to which 20% methanol (v/v) had been added (solvent A). The elution conditions for the *o*-PA derivatives were 0−50% solvent B

[2] The putative high-valent iron−oxo complex is oxidized by 2 equiv above the resting state of the ferric enzyme. In Scheme 2, the curved line represents the dianion of protoporphyrin IX; the axial ligand to the heme is a thiolate anion. Thus, the overall charge on the iron oxo complex is zero, regardless of the atomic location of the oxidizing equivalents. In Schemes 4−6, $[Fe{=}O]^{3+}$ denotes the same high-valent iron−oxo complex.

(methanol) over 9 min, followed by a linear increase to 100% methanol over the next 0.5 min, 100% methanol for 3 min, and a return to 100% solvent A over the next 0.5 min. For the NDA derivatives, the conditions were a linear increase from 0 to 100% solvent B over 12.5 min, followed by 5 min of 100% methanol, and a return to the initial conditions over the last 0.5 min. The flow rate for both methods was 0.5 mL/min. Amino acid standards were used to quantify the samples. The concentration of a stock solution of synthetic CN-orn was determined by conversion to citrulline (see below) and comparison to citrulline standards. Phenylalanine was used as an internal standard. Retention times for *o*-PA derivatives: citrulline, 5.9 min; CN-orn, 7.5 min; NHA, 7.8 min; Phe, 8.9 min. Retention times for NDA derivatives: citrulline, 7.3 min; CN-orn, 8.0 min; NHA, 9.1 min; Phe, 9.7 min.

Quantitation of NO_2^-/NO_3^-. Nitrate was reduced to nitrite and quantified by the Griess reaction and comparison to authentic standards, as described in the assay kit (Cayman Chemical Co., Ann Arbor, MI).

Enzyme Reactions: (A) H_2O_2-Dependent Reactions. Reactions were performed at 25 and 37 °C in 50 mM Hepes, pH 7.4. Reactions consisted of 100 or 200 μM NHA, 0−100 mM H_2O_2, 0.12−1.2 μM NOS, and 0 or 10 μM H_4B (with 155 μM DTT). Some reactions contained 1 mM $CaCl_2$ and/or 1.2 μM CaM; no changes in reactivity were observed. Reactions were quenched by the addition of 200 units of catalase (Sigma, C 3155) per 100 μL of reaction, where a unit is as defined by the supplier. Reactions or standards that contained NHA and were to be analyzed with NDA had 200 μM DTT added prior to HPLC analysis to prevent oxidation of NHA to CN-orn and citrulline during derivatization. Further reaction of CN-orn by nNOS was measured by substituting CN-orn (100 μM−5 mM) for NHA in nNOS reactions, with and without H_2O_2, Ca^{2+}/CaM, and NADPH.

(B) NADPH-Dependent Reactions. Reactions were performed at 25 and 37 °C in 50 mM Hepes, pH 7.4. Reactions consisted of 50−200 μM NHA or 50−200 μM arginine, 120−300 μM NADPH, 0.12−1.2 μM NOS, 1 mM $CaCl_2$, 1.2 μM CaM, 0−20 μM H_4B, and 0−300 μM DTT. Reactions were quenched with an equal volume of 10 M urea and analyzed by HPLC. As with the H_2O_2 reactions, samples containing NHA had 200 μM DTT added prior to NDA derivatization and HPLC analysis. For measuring the inhibition of arginine oxidation by CN-orn, oxyhemoglobin assays contained 5 μM oxyhemoglobin, 1 μM arginine, 1 mM $CaCl_2$, 1.2 mM CaM, 120 μM NADPH, and 73 nM nNOS, in the presence or absence of 1 mM CN-orn. These reactions were incubated at 37 °C and initiated with enzyme.

Coupled HPLC/MS. Preliminary molecular weight determinations were done by electrospray ionization on either a Hewlett-Packard 5989B mass spectrometer or a Finnigan TSQ 7000 tandem quadrupole mass spectrometer. Tandem mass spectrometry experiments (LC-MS-MS) were carried out on the TSQ 7000 in the negative-ion electrospray mode. H_2O_2-dependent reaction mixtures were derivatized with *o*-PA and 2-mercaptoethanol as described above, except that the potassium borate buffer was replaced by ammonium hydroxide to give a final concentration of about 100 mM. The same conditions were used for the standards, i.e., NHA and citrulline. The HPLC conditions were identical to those described above, with the eluate from the HPLC being split 1:1 between waste and the mass spectrometer. For collision-induced decomposition, the first quadrupole (Q1) was set to transmit *m/z* 332 and the second quadrupole (Q3) was scanned over the range *m/z* 150−350. The collision offset voltage was 15 eV and the collision gas was argon at pressures of 1.5−3 mTorr.

Synthesis and Characterization of N^{δ}-Cyano-L-ornithine: (A). Free-Base Ornithine. L-Ornithine hydrochloride (3.36 g, 20 mmol) was dissolved in minimal water (∼15 mL). One equivalent of NaOH (0.80 g) was added. After cooling, cold 95% ethanol was added to induce precipitation (∼50 mL total volume). The white precipitate was filtered and washed with ethanol and then with Et_2O. Yield: 2.6 g, 98%.

(B) N^{δ}-Cyano-L-ornithine. L-Ornithine (1.32 g, 10 mmol) was dissolved in minimal water (∼10 mL), and $CuCO_3 \cdot Cu(OH)_2$ (0.552 g, 2.5 mmol) was added with stirring and heating. The reaction mixture was filtered to remove unreacted copper carbonate, and 1,10-phenanthroline (0.99 g, 5 mmol) was added to improve the solubility of the copper complex during the following reaction. The solvent was removed by rotary evaporation under reduced pressure from a 500-mL round-bottom flask. To ensure the removal of all the water, several aliquots of absolute ethanol were added and removed. The copper complex was dissolved in ∼ 250 mL of methanol (which had been dried over 3 Å molecular sieves). A solution of cyanogen bromide (12 mmol, 1.27 g) in methanol (40 mL) was added dropwise over 30 min (*4*) with stirring to the ice-cooled copper complex. The reaction was allowed to proceed overnight. The solvent was removed and 10 mL of water was added to give a colorless solution and a green precipitate. The precipitate was filtered and washed with additional water (∼10 mL). The aqueous phase was neutralized with solid ammonium bicarbonate and then acidified to pH ∼4 with aqueous sodium acetate buffer. The resulting blue solution was applied to a Chelex column to remove the copper ions. The Chelex column was made from 30 g of Chelex that was washed with 3 bed volumes of 0.5 M sodium acetate (pH 4.0) and 10 bed volumes of water. The amino acids were visualized on silica TLC plates with methanol as the mobile phase by reaction with ninhydrin spray reagent ($R_f = 0.34$ for citrulline; $R_f = 0.60$ for CN-orn; ornithine was immobile). Fractions that contained amino acids were evaporated in the presence of silica gel and applied to the top of a methanol-equilibrated silica column (1 cm × 12 cm). CN-orn was eluted with methanol. One milliliter of 200 mM sodium acetate (pH 4.0) was added to the pooled CN-orn fractions, and the methanol was removed by rotary evaporation. Attempts to isolate CN-orn resulted in polymerization, as evinced by the loss of reactivity with ninhydrin. The aqueous solution of CN-orn was stored at −80 °C. Yield: 16%. Purity was estimated to be >95% by TLC. The ^{13}C NMR spectrum was recorded at 125 MHz with a Bruker Avance DRX-500 instrument on a 160 mM sample of CN-orn with 170 mM NaOAc buffer, with 15% D_2O for the signal lock, a 3-s delay between pulses, and dioxane as an internal standard (δ 66.5 ppm). CN-orn: δ 173.4, 118.7, 44.6, 41.5, 27.6, and 20.6 ppm. The IR spectrum was measured with a Nicolet 60-SX FT-IR. CN-orn exhibited an intense band at 2220 cm^{-1} in aqueous solution.

Nonenzymatic Conversion of CN-orn to Citrulline. CN-orn (100 μM−5 mM) was allowed to react at 25 and 37 °C

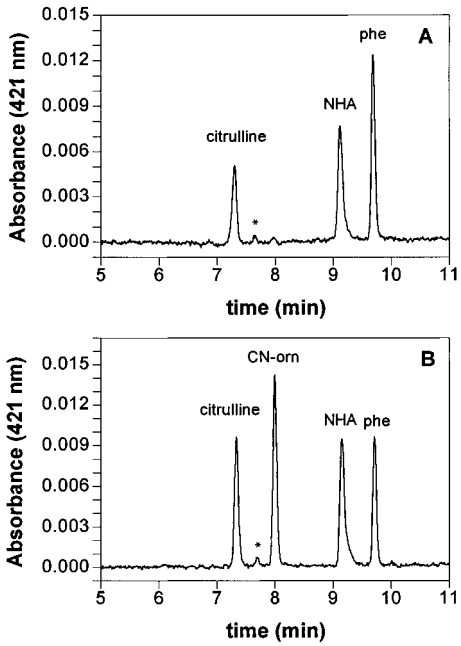


FIGURE 1: HPLC traces of the NDA-derivatized reactions of NHA and NOS. (A) NADPH- and O_2-dependent reaction. (B) H_2O_2-dependent reaction. Reaction conditions were (for A) 100 μM NHA, 100 μM phenylalanine, 1.2 μM NOS, 300 μM NADPH, 1 mM $CaCl_2$, 1.2 μM CaM, 17 μM H_4B, 230 μM DTT, and 50 mM Hepes (pH 7.4) and (for B) 200 μM NHA, 100 μM phenylalanine, 1.3 μM NOS, 100 mM H_2O_2, 1 mM $CaCl_2$, 20 μM H_4B, 280 μM DTT, and 50 mM Hepes (pH 7.4). Reactions were run at 25 °C, for 1 and 10 min, respectively. The asterisk at 7.7 min marks trace amounts of glycine in the buffers.

for up to 12 h to determine, by HPLC, whether it was converted to citrulline.

Hydrogen Peroxide Determination. The hydrogen peroxide concentration was determined by measuring the triiodide (I_3^-; $\lambda_{max} = 353$ nm, $\epsilon = 26\,400$ M^{-1} cm^{-1}) formed by the chloroperoxidase-catalyzed oxidation of iodide by hydrogen peroxide (*27*).

RESULTS

Amino Acid Products of NADPH- and H_2O_2-Dependent Reactions. The HPLC chromatograms of two NDA-derivatized nNOS reactions with NHA are shown in Figure 1. The NADPH- and O_2-dependent reaction is shown in panel A. The peak at 7.3 min is the product citrulline, formed at the expense of NHA (retention time 9.1 min). Phenylalanine served as an internal standard and eluted at 9.7 min. Panel B is the chromatogram of the H_2O_2-dependent reaction. In addition to citrulline, NHA, and phenylalanine, another peak was observed at 8.0 min. The new product was identified as CN-orn (see below). The formation of this product was time-dependent (data not shown) and required H_2O_2, NHA, and enzyme. The Ca^{2+}/CaM complex is required for NADPH-dependent turnover because its binding to nNOS is necessary for intramolecular electron transfer. Since oxygen is already reduced in the H_2O_2 reactions, Ca^{2+}/CaM should not have any effect on the reaction. In fact, the chromatograms for H_2O_2-dependent reactions with CaM or without $CaCl_2$ are identical to that in Figure 1B. Enzymatic oxidation of arginine resulted in the exclusive conversion to citrulline during NADPH turnover; a small amount of NHA was also released from the enzyme during turnover (not shown). nNOS did not catalyze the oxidation of arginine by H_2O_2. In the absence of nNOS, prolonged incubations of NHA and H_2O_2 resulted in small amounts (2%) of citrulline and the unidentified product.

Assignment of the New Product as CN-orn by HPLC/MS. The products from the H_2O_2-dependent reaction were initially characterized by negative-ion LC-MS-MS analysis of their *o*-PA derivatives. The $[M - H]^-$ ions of the new product and of citrulline were observed at *m/z* 332 and 350, corresponding to molecular weights of 333 and 351, respectively. This difference of 18 Da is consistent with a formula of $C_{16}H_{18}N_3O_3S$ for the $[M - H]^-$ ion of the new product. Collision-induced decomposition of *m/z* 332 produced major fragments at *m/z* 192 and 288. The fragment at *m/z* 192 is consistent with the 2-mercaptoethanol-substituted isoindole moiety, while that at *m/z* 288 can arise from decarboxylation of the CN-orn/*o*-PA derivative. In an identical experiment performed with ^{15}N-labeled NHA, in which the isotope label is specifically incorporated as the hydroxy-bearing guanidino nitrogen (*4*, *28*), no label incorporation is observed in either of the reaction products, citrulline or the new product.

Identification of the New Product as CN-orn. The identity of the second enzymatic product was confirmed by synthesis of CN-orn and comparison of the synthetic and enzymatic materials. CN-orn was synthesized from the copper complex of ornithine, using cyanogen bromide as the source of the cyano functionality (*4*, *28*) and copper(II) complexation as a way to reduce the nucleophilicity of the α-amino and α-carboxy groups of ornithine (*29*). The ^{13}C NMR resonance at 118.7 ppm and the IR stretch at 2220 cm^{-1} (H_2O) confirm the presence of the cyanamide functionality in the synthetic material (*30*, *31*). The NDA and *o*-PA derivatives of synthetic CN-orn elute from the HPLC column with identical retention times to the derivatives of the product formed during hydrogen peroxide turnover. Coinjection of synthetic and enzymatic material results in a single, normally shaped peak. Finally, the hydration of CN-orn can be readily effected by incubation with 1 M 2-mercaptoethanol in potassium borate buffer (pH 10.4) for 10 min, to produce citrulline as the sole amino acid. These conditions are similar to those used for *o*-PA derivatization. Once the identity of the new product was established, it became evident that some CN-orn was being converted to citrulline during the *o*-PA derivatizations. Conditions such as 0.6 M potassium borate (pH 10.4), 0.1 M NaOH, 0.1 M HCl, or 0.12 M TCA resulted in some conversion to citrulline. The instability of CN-orn with respect to conversion to citrulline and to polymerization is consistent with previous observations (*28*, *32*). Virtually no conversion of CN-orn to citrulline occurs during NDA derivatization and analysis; however; NHA is susceptible to oxidation during the longer derivatization reaction. Addition of 200 μM DTT decreases the signal intensity somewhat (~35%) but prevents the oxidation of NHA. Further support for the assignment of the enzymatic product as CN-orn comes from a comparison of the product distribution observed with the two derivatization methods: only a small fraction of the product is observed as CN-orn in the *o*-PA derivatization (data not shown), while the milder conditions of the NDA derivatization reveal that approximately half the product is CN-orn (Figure 1B).

Stoichiometry of Amino Acid to Inorganic Products. The formation of amino acid and inorganic products from NHA and H_2O_2 was determined as a function of nNOS concentration (Figure 2). The amino acid values are the sum of the

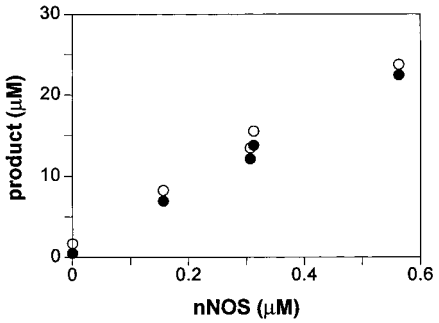


FIGURE 2: Formation of amino acids and NO_2^-/NO_3^- as a function of nNOS concentration. The sum of the concentrations of CN-orn and citrulline are shown with ○ symbols and the concentration of NO_2^- and NO_3^- is shown by ●. Reactions consisted of 100 μM NHA, 0−0.56 μM NOS, 10 μM H_4B, 155 μM DTT, 38 mM H_2O_2, and 50 μM Hepes (pH 7.4). Reactions were incubated at 37 °C for 4 min, quenched with catalase, and divided for analysis of amino acids and NO_2^-/NO_3^-, as described under Experimental Procedures. The values shown for the amino acids are averages of duplicate analyses.

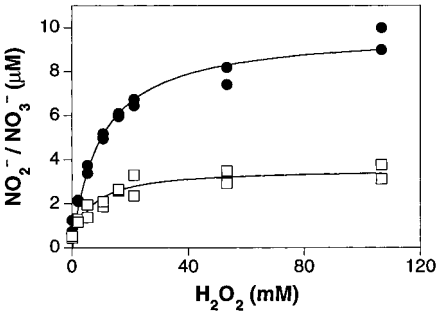


FIGURE 3: Formation of NO_2^-/NO_3^- as a function of hydrogen peroxide concentration. Reactions consisted of 100 μM NHA, 0.31 μM NOS, 0−105 mM H_2O_2, and 50 mM Hepes (pH 7.4). Reactions with H_4B also contained 10 μM H_4B and 155 μM DTT. Reactions were incubated at 37 °C for 2 min (no added H_4B) or 4 min (with H_4B) and quenched with catalase. The experimental data are designated with symbols (□, no added H_4B; ●, 10 μM H_4B). The solid lines are best fits to the data (values in text).

values obtained for CN-orn and citrulline. The stoichiometry of amino acid to NO_2^-/NO_3^- is 1.0 ± 0.1. The samples were derivatized with *o*-PA. Quantitation of the *o*-PA derivatives indicated that the concentrations of all the amino acids present summed to the original concentration of substrate. Together with this demonstration of mass balance, data from NDA derivatization were used to determine that the CN-orn concentration is 50% ± 5% of the amino acid products. The remainder is citrulline.

Kinetics of H_2O_2-Dependent Oxidation of NHA. The dependence of NO_2^-/NO_3^- formation on the concentration of H_2O_2 was determined for the nNOS-catalyzed reaction in the presence and absence of additional H_4B. The reactions were run at 37 °C and quenched with catalase after 4 and 2 min, respectively. Product formation was linear with time over these intervals (data not shown). The results are shown in Figure 3. The data were fit to simple saturation kinetics (PSI-Plot). The apparent kinetic constants are $K_{m,app}(H_2O_2) = 6 \pm 1$ mM and $V_{max} = 36 \pm 2$ nmol min^{-1} mg^{-1} for the reactions without added H_4B and $K_{m,app}(H_2O_2) = 10 \pm 1$ mM and $V_{max} = 49 \pm 2$ nmol min^{-1} mg^{-1} for the reactions with 10 μM added H_4B.

Role of CN-orn in Catalysis. CN-orn did not cause a spectral shift in the Soret band of nNOS at concentrations up to 600 μM (data not shown). At a CN-orn concentration of 1 mM, the NADPH-dependent oxidation of arginine (1 μM) to citrulline and •NO was inhibited by only 35%, as measured by the oxyhemoglobin assay (data not shown). Incubation of CN-orn (100 μM−5 mM) with nNOS did not result in conversion to citrulline under conditions of NADPH- or H_2O_2-dependent catalysis (data not shown). On the basis of the above, CN-orn appears not to bind significantly to nNOS nor to be a precursor of citrulline.

Oxidants Other Than Hydrogen Peroxide. The enzymatic oxidation of NHA was assayed with several obligate oxygen-atom donors. No enzymatic reaction was observed with 200 μM NHA and 10 mM *tert*-butyl hydroperoxide, potassium hydropersulfate (Oxone), magnesium perphthalate, or peracetic acid (data not shown).

DISCUSSION

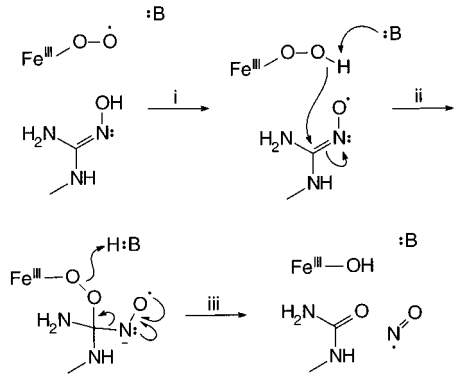
When hydrogen peroxide is used as an oxidant, nNOS catalyzes the formation of CN-orn and citrulline from NHA. By contrast, with NADPH and oxygen, nNOS catalyzes the exclusive formation of citrulline from either NHA or arginine. The reaction of H_2O_2 and NHA with inducible NOS (iNOS) was studied previously (*20*). The H_2O_2-dependent reaction of iNOS proceeded at a rate comparable to that of the NADPH-dependent oxidation of NHA. Citrulline was the only amino acid product observed, although its formation was substoichiometric with respect to the formation of NO_2^- and NO_3^- under aerobic conditions. Since the HPLC method developed for this report provided much greater resolution, we decided to reexamine the reaction of iNOS with NHA and H_2O_2 and found that CN-orn is a major product along with citrulline (Spanbauer and Marletta, unpublished observations).

We considered three possibilities to explain the formation of CN-orn: (i) that CN-orn is formed as an intermediate on the NADPH-dependent pathway to citrulline, much as NHA is an intermediate in arginine oxidation to citrulline; (ii) that citrulline and CN-orn are formed from a common intermediate in the NADPH-dependent reaction; and (iii) that the use of hydrogen peroxide causes another mechanism to be catalytically relevant, which results in the formation of CN-orn in addition to citrulline.

If CN-orn were an intermediate in the NADPH-dependent transformation of arginine to citrulline (possibility i), it would be expected to show substrate-like behavior. However, CN-orn did not cause a blue shift in the Soret maximum, which is often observed upon substrate binding to NOS and other P450s, nor was it enzymatically converted to citrulline. Further, inhibition of arginine oxidation might be expected from competition between arginine and CN-orn for the active site of the enzyme, but CN-orn only weakly inhibited •NO formation from arginine. By contrast, NHA does cause a spectral binding shift in the position of the Soret band and functions as a substrate in normal catalysis. Thus, no evidence supports the hypothesis that CN-orn is an intermediate in the nNOS reaction.

Alternatively, as stated in (ii) above, citrulline and CN-orn could be formed from a common intermediate during NADPH-dependent catalysis. For example, if the diimino tautomer of CN-orn were initially formed, then it could undergo tautomerization or hydration to yield CN-orn or citrulline, respectively. This mechanism is consistent with the inability of CN-orn to function as a substrate for NOS.

Scheme 3: Proposed Mechanism for the Formation of Citrulline and Nitric Oxide from NHA, NADPH, and O_2

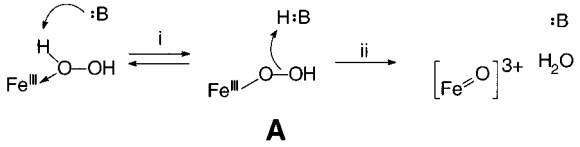


However, this mechanism requires an explanation of why a putative diimino intermediate would decompose to a single product in the NADPH and O_2 reaction but partition between two products in the H_2O_2 reaction. Although we can not conclusively eliminate this mechanism, we consider it less plausible than a mechanism in which hydrogen peroxide generates a different oxidant from the one generated during NADPH-dependent catalysis (possibility iii). The formation of oxidizing intermediates has been studied in NOS and other heme enzymes. The mechanisms described below are derived from precedents from both the chemical and biochemical literature.

Scheme 2 is a modified P450 cycle (*33*). Reduction of the ferric state of the enzyme (upper right) with an NADPH-derived reducing equivalent followed by O_2 binding yields the resonance structures shown at the bottom. A second reduction yields a putative ferric hydroperoxo complex, which can oxidize substrate directly or undergo O−O bond scission prior to substrate oxidation. The basis for the peroxide shunt is that the two-electron reduction of molecular oxygen produces an oxygen species at the oxidation level of peroxide. For enzymatic reactions in which O−O bond scission [i.e., (per)ferryl formation] precedes substrate oxidation, the peroxide adduct is proposed to undergo O−O bond scission to yield the same iron−oxo intermediate that is obtained from molecular oxygen (*33*). The iron−oxo intermediate oxidizes substrates by 2 equiv. However, the conversion of NHA to citrulline and •NO is a three-electron oxidation, which has been hypothesized to occur by the one-electron oxidation of NHA by the ferric superoxo complex (which completes the reduction of O_2 to the level of peroxide) followed by the two-electron oxidation of the NHA radical by peroxide. Although the later steps of this mechanism are speculative for NOS, spectrophotometric data confirm the stability of ferric NOS in the presence of NHA, the requirement for reducing equivalents from NADPH to reduce the heme, and sensitivity of ferrous NOS to molecular oxygen (*19*).

The detailed proposal for NHA oxidation by NADPH and O_2 is that NADPH contributes only one reducing equivalent per turnover. That reducing equivalent reduces ferric enzyme to the ferrous state. Addition of O_2 to ferrous enzyme yields $[Fe^{II}(O_2)] \leftrightarrow [Fe^{III}(O_2^{\bullet -})]$, the first structure of Scheme 3. Hydrogen-atom abstraction from NHA generates the ferric hydroperoxo complex and an NHA radical (Scheme 3, reaction i). Nucleophilic addition of peroxide to the guanidino carbon (reaction ii) produces a putative, tetrahedral intermediate, which decomposes to citrulline and •NO (reaction iii) (*2*, *34*). The collapse of this tetrahedral intermediate to the observed products, citrulline and •NO, can occur by proton transfer (reaction iii), hydride transfer, or single-electron chemistry. Nearly the same mechanism can be envisioned for citrulline and NO^- formation from H_2O_2 and NHA. The difference in oxidizing equivalents (two instead of three) alters reaction ii in Scheme 3 so that NHA (rather than its radical) undergoes nucleophilic addition by peroxide. The expected product NO^- is detected as NO_2^- and NO_3^-; its oxidation under aerobic conditions has been discussed (*20*). The mechanism of CN-orn formation will be addressed below.

Scheme 4: Proposed Mechanism for the Reaction of Hydrogen Peroxide with nNOS



A

In addition to the difference in oxidizing equivalents of a H_2O_2-derived species, the protonation state may be greater than that obtained from electron transfer to O_2. As shown in Scheme 4, the protons of hydrogen peroxide may provide an accessible pathway for O−O bond scission. The active-site base, which in Scheme 3 served to increase the nucleophilicity of the iron-bound hydroperoxide, may instead serve to deprotonate the incoming peroxide (Scheme 4, reaction i). Heterolytic O−O bond scission is facilitated by protonation of the *other* oxygen atom (reaction ii). The high-valent iron−oxo complex thus formed is oxidized by 2 equiv relative to ferric enzyme. The activation of dioxygen and hydrogen peroxide by NOS has important precedents in the reactions of steroidal P450s, synthetic heme complexes, and peroxidases.

Some P450 enzymes appear to oxidize electrophilic substrates with a ferric peroxo complex derived from NAD(P)H and O_2. Studies of the oxidation of isotopically labeled estrogen precursors by a microsomal preparation of human placenta demonstrated that the C-19 methyl group is oxidized to formate with incorporation of molecular oxygen in both the methyl-to-aldehyde and aldehyde-to-formate steps. Akhtar and co-workers proposed that the C-19 methyl group is oxidized to the alcohol and then the aldehyde in two classical perferryl oxidations. Based on the labeling data, the mechanism of the third oxidative step was proposed to involve addition of a nucleophilic peroxide to the aldehyde. The mechanism was proposed to change from iron−oxo to iron−peroxo because of the increased electrophilicity of the aldehyde substrate compared with the methyl and the alcohol (*35*). Biochemical evidence supporting a nucleophilic peroxide oxidant was obtained with cytochrome P450 LM_2 (CYP2B4): among a variety of alternative oxidants, only H_2O_2 is able to substitute for NADPH and O_2 in the deformylation/desaturation of cyclohexane carboxaldehyde to cyclohexene (*36*). In a related chemical reaction, a 19-aldehyde, steroidal diene undergoes deformylation and aromatization in a reaction that requires H_2O_2 and is accelerated by KOH. The kinetics of this reaction is consistent with the formation, from the aldehyde, of a tetrahedral hydroxy hydroperoxy intermediate (*37*).

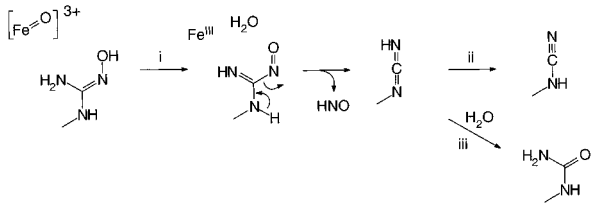
In an apparently contrary example, heme oxygenase has been shown to catalyze the oxidation of heme by H_2O_2 (or EtOOH) via a hydroperoxo (or ethylperoxo) intermediate that is certainly *not* nucleophilic: it is proposed to react by either an electrophilic or a radical mechanism (*38*). Neither of these possibilities seems likely to be relevant for NOS because the axial thiolate effectively transfers electron density to the distal oxygen.

When hydrogen peroxide undergoes bond scission with heme enzymes and model compounds, it nearly always reacts by a heterolytic pathway, in which it serves as an oxygen-atom donor (*39*, *40*). Although homolytic cleavage of the O−O bond to yield $[Fe^{IV}{=}O]^{2+}$ and an organic radical is known for reactions of peracids and organic peroxides with heme enzymes and model complexes, this mechanism requires an aprotic,[3] nonpolar environment that is unlikely to be found at the active site of NOS (*39*−*42*). Valentine and co-workers have studied the chemistry of peroxide bound to a series of ferric porphyrins in acetonitrile.[3] The epoxidation of electron-poor, but not electron-rich, olefins supports the proposed nucleophilic character of ferric peroxo complexes. The transition state for epoxidation was proposed to be an open structure (see Scheme 4, structure A) rather than a symmetrical, triangular η^2-peroxo complex as is postulated for the ground-state structure (*43*). The presence of the electron-donating thiolate ligand in NOS should further enhance the nucleophilicity of the peroxide ligand.

Cytochrome *c* peroxidase (CcP) activates H_2O_2 by catalyzing a 1,2-hydrogen shift and heterolytic cleavage (Scheme 4). Extensive biochemical and structural studies have identified the active-site residues involved in the initial, rate-limiting deprotonation of hydrogen peroxide to form Fe^{III}-OOH (*47*−*49*). The electron-donating axial ligand (histidine imidazolate) results in electron donation into the σ^* orbital of the peroxide, which weakens the O−O bond, and in stabilization of the electron-deficient, iron-bound oxygen (*42*, *50*). The amino acid side chains in the active site of NOS are not yet known. Nevertheless, if an active-site base is oriented to deprotonate the hydroperoxide formed by hydrogen-atom abstraction from NHA (Scheme 3, reaction ii), it might be poorly positioned to deprotonate the incoming hydrogen peroxide at the coordinating oxygen (Scheme 4, reaction i). A slightly different position of this putative base could also account for the faster rate of H_2O_2-dependent catalysis by iNOS compared with nNOS.

On the basis of chemical and biochemical precedent, the reactivity of a heme iron−peroxo intermediate (especially one with an axial thiolate ligand) seems to depend on two competing factors: (i) a proton relay, which accelerates O−O bond scission, and (ii) an electrophilic substrate, which may divert the bound peroxide from O−O bond scission to nucleophilic addition. From the crystal structure of cytochrome $P450_{cam}$, an active-site threonine was identified as a putative proton source (*51*). Vaz and Coon investigated the role of the analogous active-site threonine (Thr-302) in facilitating O-O bond scission in P450 2B4 (CYP2B4) (*52*). This enzyme catalyzes classical hydroxylations as well as deformylation/desaturations. Mutation of Thr-302 to alanine results in a decrease in the rate of hydroxylation reactions and a dramatic *increase* in the rate of deformylation reactions. The differential effects of the mutation implicate two oxidative mechanisms, i.e., one that requires O−O bond scission, and thus Thr-302, and another that requires that the O−O bond remain intact, and thus is favored by mutation of Thr-302 and the loss of a proton source.

Scheme 5: Oxidation of NHA via a Nitrosoamidine



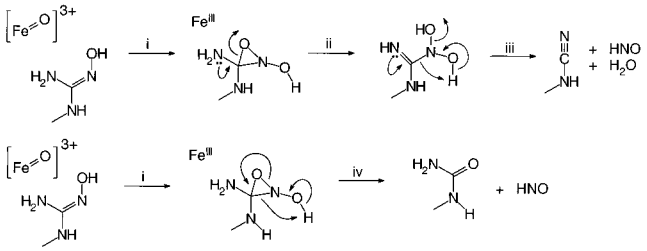
The mechanistic details of NHA oxidation are still unclear. In reactions analogous to the one described here, chemical oxidation of amidoximes has been reported to yield the corresponding nitriles and amides and NO_2^-/NO_3^- (under aerobic conditions), while the oxidation of *N*-hydroxyguanidines yields cyanamides and ureas and $^{\bullet}NO$ or NO^- (*53*−*55*). Several mechanisms account for the formation of citrulline and NO^-: electrophilic peroxidation, homolytic cleavage of the O−O bond, and oxygen rebound, as well as nucleophilic peroxidation. It is the formation of CN-orn that necessitates the formulation of a new mechanism.

We propose that nNOS and H_2O_2 react by a 1,2-hydrogen shift, and the concomitant loss of water, to produce a high-valent iron−oxo species,[2] which serves as an active oxidant (Scheme 4). Several mechanisms of oxidation can then be envisioned that account for the products. The iron oxo complex could oxidize NHA by two electrons (peroxidase chemistry, Scheme 5, reaction i), leading to the formation of a nitrosoamidine. This nitrosoamidine could decompose by loss of NO^- to a diimino intermediate, which would undergo tautomerization to CN-orn (reaction ii) or hydration to citrulline (reaction iii) (*56*). The incorporation of ^{18}O from $H_2{}^{18}O_2$ into citrulline (*20*) requires that the water incorporated in reaction iii be derived from H_2O_2, not from solvent.[4] The drawback of this mechanism is that peroxidase reactions generally occur at the heme edge with substrates that have limited access to the heme iron. Studies of ^{18}O incorporation from $^{18}O_2$ and $H_2{}^{18}O_2$ into citrulline suggest that NHA has good access to the heme iron (*20*, *57*), so monooxygenase chemistry seems more likely. Hydroxylation of the other guanadino nitrogen would form N^G,$N^{G\prime}$-dihydroxyarginine, which could lose H_2O and HNO to form CN-orn. We showed, however, that the hydroxy-bearing nitrogen is not retained in CN-orn, so this mechanism requires that the symmetric N^G,$N^{G\prime}$-dihydroxyarginine dehydrate asymmetrically. We favor a mechanism that does not require such specificity in a nonphysiological reaction. Oxygen-atom transfer from the perferryl species to the oxime could produce an oxaziridine intermediate (Scheme 6, reaction i), which

[3] Heterolytic O−O bond scission of peroxides and peracids has been shown to be catalyzed by protic solvents (*39*) and by proton donation from a second equivalent of peracid (*44*). Ab initio calculations also support the importance of solvent in lowering the activation barrier for O-atom transfer from hydrogen peroxide (*45*). An elegant model complex, in which the heme has an appended alkyl thiolate as an axial ligand to iron, is claimed to undergo heterolytic O−O bond scission under *aprotic* conditions, despite the use of a 10-fold molar excess of peracid (*46*). By contrast, the stability of the O−O bond in a series of heme−peroxo complexes is due to their formation from potassium superoxide accompanied by the rigorous exclusion of water (*43*).

[4] The ^{18}O labeling studies used iNOS, not nNOS. This analysis assumes the labeling pattern is the same for the two isoforms.

Scheme 6: Oxidation of NHA via an Oxaziridine



would decompose to CN-orn, HNO, and H_2O (reactions ii and iii) or to citrulline and HNO (reaction iv) (*56*). This oxaziridine mechanism seems to be the most likely pathway to the observed products.

The results presented here demonstrate that nNOS catalyzes the formation of citrulline and CN-orn from NHA and H_2O_2. Further, NO_2^- and NO_3^- are formed in stoichiometric amounts with citrulline and CN-orn. Citrulline may be formed in the H_2O_2-dependent reaction by essentially the same mechanism as it is in the NADPH- and O_2-dependent reaction (Scheme 3), in which a ferric peroxo complex undergoes nucleophilic addition to NHA (or its radical). Alternatively, citrulline may be formed in a single oxidative step by a high-valent iron−oxo complex (Scheme 6, reactions i and iv). Although citrulline may be formed by two mechanisms, the formation of CN-orn is best rationalized by oxidation of NHA by an iron−oxo complex. The putative iron−oxo complex probably forms CN-orn via an oxaziridine by O-atom insertion (Scheme 6, reactions i−iii).

This work exemplifies the risk in the common assumption that a substitution such as H_2O_2 for NADPH and O_2 has a negligible effect on the mechanism. Despite the change in mechanism, the current study enhances our understanding of the normal mechanism. Without the data from the H_2O_2-dependent reaction, one can envision NHA oxidation by $Fe^{III}(O_2^{\bullet -})$ to give NHA$^\bullet$ and Fe^{III}(OOH), which could undergo O−O bond scission prior to further substrate oxidation. Because the additional proton in the H_2O_2-dependent reaction greatly facilitates this pathway, it appears highly unlikely that the iron−oxo pathway would be relevant in the NADPH-dependent reaction, where CN-orn is not observed. Thus, we can assert with greater confidence that the O−O bond is intact during the second part of NHA oxidation in NADPH-dependent catalysis (Scheme 3).

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SUPPORTING INFORMATION AVAILABLE

Two figures showing mass spectra of the 7.5-min HPLC peak from the reaction of nNOS, NHA, and H_2O_2, derivatized with *o*-PA (electrospray ionization and collision-induced decomposition) (2 pages). Ordering information is available on any current masthead page.

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