Characterization of the Inactivation of Nitric Oxide Synthase by N^G-Methyl-L-arginine: Evidence for Heme Loss†

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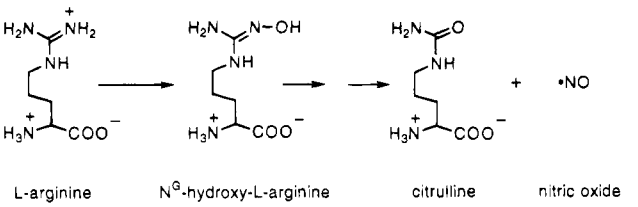
ABSTRACT: The nitric oxide synthases (NOS) are a unique family of P450-type hemoproteins that catalyze the formation of •NO and citrulline from L-arginine, oxygen, and NADPH. N^G-Methyl-L-arginine (L-NMA) has been shown to function as a slow, partially uncoupled alternate substrate and mechanism-based inhibitor of the inducible NOS [Olken, N. M., & Marletta, M. A. (1993) *Biochemistry 32*, 9677−9685]. In this report, the inactivation of NOS by L-NMA has been investigated in detail. Inactivation fails to occur under an argon atmosphere, establishing turnover dependence. The partition ratio, defined as the number of molecules of citrulline formed per NOS monomer inactivated, is 108 ± 3. By utilizing N^G-methyl-L-[2,3-$^3H_2$]arginine and N^G-[^{14}C]methyl-L-arginine, the stoichiometry of radiolabeling is 0.11 ± 0.01 equiv of tritium and 0.41 ± 0.10 equiv of carbon-14 per inactivated NOS monomer. Dialysis under native conditions does not change this stoichiometry. However, dialysis of NOS following denaturation decreases the stoichiometry of radiolabeling to 0.08 ± 0.04 equiv of tritium and 0.12 ± 0.04 equiv of carbon-14 per inactivated NOS monomer. Absolute and CO-reduced difference spectroscopy indicates that inactivation of L-NMA is accompanied by a substantial loss of the heme chromophore, which is not prevented by catalase. HPLC analysis of NOS heme following inactivation with L-NMA indicates substantial loss of heme. These findings suggest that multiple mechanisms may contribute to the loss of NOS activity by L-NMA, including heme loss and possibly protein and cofactor modification.

The nitric oxide synthases (NOS)[1] are cytochrome P450-type hemoproteins that catalyze the formation of nitric oxide (•NO) and citrulline from L-arginine, O_2, and NADPH (Scheme 1) (Marletta, 1993; Nathan, 1992). The inducible murine macrophage NOS is active as a homodimer (Hevel et al., 1991; Stuehr et al., 1991a) and has been shown to be purified with 1 equiv each of tightly bound FAD, FMN (Hevel et al., 1991), and iron protoporphyrin IX (Fe-PPIX) (Stuehr & Ikeda-Saito, 1992; White & Marletta, 1992) per monomer. 6(*R*)-Tetrahydro-L-biopterin (H_4B) has also been shown to be bound to NOS following purification, although in variable and substoichiometric amounts (Hevel & Marletta, 1992; Mayer et al., 1991; Schmidt et al., 1992). When purified in the presence of H_4B and reconstituted after purification, the murine inducible NOS contained one bound H_4B per subunit (Hevel & Marletta, 1992). The high degree of sequence homology at the carboxy-terminal region of NOS to NADPH-cytochrome P450 reductase has suggested that the flavins are organized into an NADPH-cytochrome P450 reductase domain (Bredt et al., 1991). The amino-terminal region of NOS presumably contains the heme-binding domain, yet it bears little sequence homology to members of the P450 superfamily (Nelson et al., 1993). However, the inducible and constitutive NOS isoforms have been classified as P450-type hemoproteins primarily because the reduced-CO complex of each displays the characteristic absorbance at about 450 nm (McMillan et al., 1992; Stuehr & Ikeda-Saito, 1992; White & Marletta, 1992). Resonance Raman spectroscopy of the constitutive rat neuronal NOS was consistent with a thiolate axial ligand to the heme iron (Wang et al., 1993).

Scheme 1: Reaction Catalyzed by the Nitric Oxide Synthases



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[1] Abbreviations: NOS, nitric oxide synthase; L-NMA, N^G-methyl-L-arginine; L-NHA, N^G-hydroxy-L-arginine; L-NHMA, N^G-hydroxy-N^G-methyl-L-arginine; [^{3}H]-L-NMA, N^G-methyl-L-[2,3-$^3H_2$]arginine; [^{14}C]-L-NMA, N^G-[^{14}C]methyl-L-arginine; oxyHb, ferrous oxyhemoglobin; metHb, ferric hemoglobin; H_4B, 6(*R*)-tetrahydro-L-biopterin; DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RP-HPLC, reverse-phase high-performance liquid chromatography; t_r, retention time; FAD, flavin adenine dinucleotide; FMN, flavin mononucleoside; SOD, superoxide dismutase; P450, cytochrome P450; Fe-PPIX, iron protoporphyrin IX or heme.

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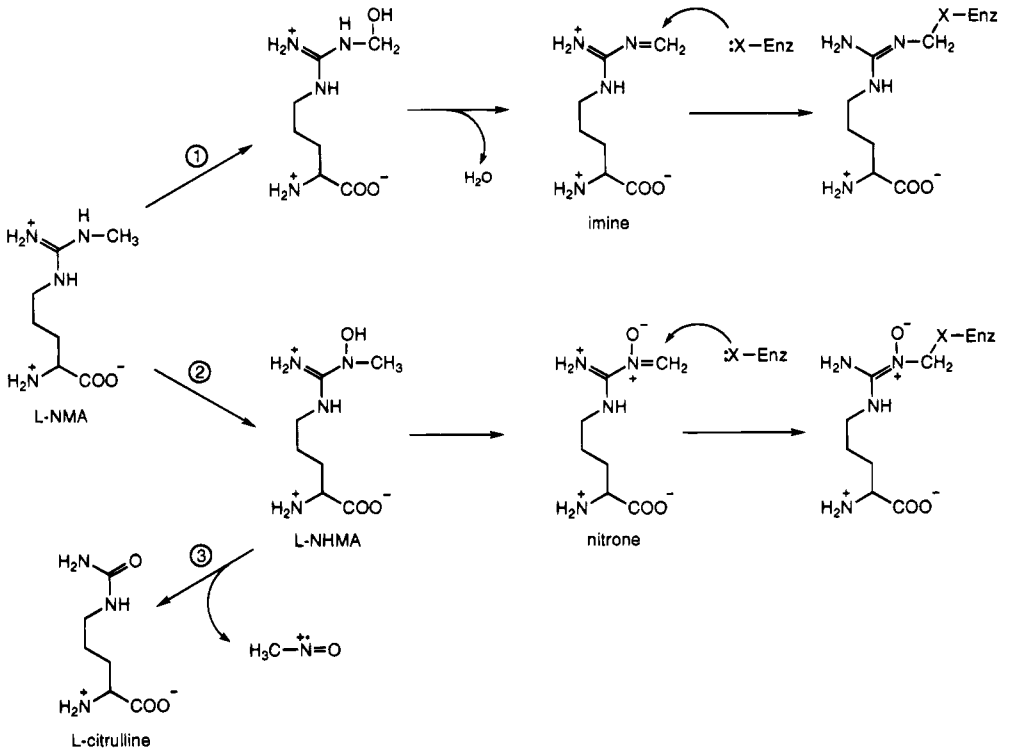
Scheme 2: L-NMA Processing and Possible Intermediates That Could Inactivate NOS[a]



[a] *C*-Hydroxylation produces a carbinolamine, which decomposes to HCHO and L-arginine but is also in equilibrium with an iminium ion, which could act as a Michael acceptor (pathway 1). *N*-Oxygenation forms L-NHMA, which is subsequently demethylated to L-NHA and HCHO, possibly via a nitrone, a potential Michael acceptor (pathway 2). Attack of L-NHMA by an enzyme-bound ferric peroxide, analogous to that proposed for L-NHA (Marletta, 1993), would result in the formation of a nitrosomethane radical cation (pathway 3), a highly reactive species that could modify active site cofactors or amino acid residues.

The mechanism of NOS has not been established, but one step in the reaction involves an NADPH-dependent hydroxylation of L-arginine to form L-NHA (Scheme 1) (Marletta et al., 1988; Pufahl et al., 1992; Stuehr et al., 1991b). It is not known whether the heme or the pterin catalyzes this hydroxylation, although the overall conversion of L-arginine to citrulline and NO is inhibited by CO (McMillan et al., 1992; Pufahl & Marletta, 1993; Stuehr & Ikeda-Saito, 1992; White & Marletta, 1992). L-NHA remains bound at the active site and undergoes an NADPH-dependent, regiospecific oxidation at the hydroxy-bearing guanidino nitrogen to yield •NO and citrulline (Pufahl et al., 1992; Stuehr et al., 1991b). This step in the reaction is also inhibited by CO, suggesting an involvement for the heme here as well (Pufahl & Marletta, 1993). The three-electron oxidation of L-NHA to citrulline and •NO does not appear to be a straightforward P450 reaction. It has been suggested that the L-NHA radical cation could undergo a nucleophilic attack at the guanidino carbon by an enzyme-bound ferric peroxide (Marletta, 1993), analogous to the mechanism proposed for aromatase (Akhtar et al., 1982). This tetrahedral intermediate could collapse to form •NO, citrulline, and H_2O, leaving the heme in the ferric oxidation state. Both steps of the reaction could, in principle, be carried out by the heme, leaving the redox role of the tightly bound H_4B uncertain.

L-NMA has been shown to be processed in a fashion consistent with established P450 chemistry and the overall catalytic sequence of NOS (Scheme 2) (Olken & Marletta, 1992, 1993). L-NMA processing is initiated with either *C*- or *N*-hydroxylation at the methyl-bearing guanidino carbon or nitrogen. The detection of citrulline, L-NHMA, L-arginine, and L-NHA in reaction mixtures of NOS and L-NMA is consistent with oxidation at these positions. Hydroxylation at the methyl carbon results in the formation of a carbinolamine. Collapse of this species liberates HCHO and L-arginine, both of which have been detected (Olken & Marletta, 1993). Dehydration of the carbinolamine would produce an iminium ion, which could act as a Michael acceptor of an active site nucleophile (Scheme 2). The major amino acid products detected from L-NMA were the *N*-oxygenation product, L-NHMA, and citrulline (Feldman et al., 1993; Olken & Marletta, 1992, 1993). L-NHMA was shown to be demethylated by NOS to L-NHA and HCHO, possibly by oxidation to the nitrone (a potential Michael acceptor) followed by hydrolysis (Olken & Marletta, 1993). In fact, L-NHMA, like L-NMA, was shown to function as a partially uncoupled, slow alternate substrate (Olken & Marletta, 1993) and a mechanism-based inhibitor (Feldman et al., 1993; Pufahl et al., 1992) of purified macrophage NOS. NOS was found to form •NO and citrulline from L-NMA and L-NHMA at about 5% and 10% of the initial rate, respectively, relative to L-arginine (Olken & Marletta, 1993). In contrast to the relatively tight coupling of NADPH oxidation by NOS during the processing of L-arginine or L-NHA, about 70% of the NADPH consumed during L-NMA or L-NHMA processing was shown to result in H_2O_2 formation (Olken & Marletta, 1993). However, neither H_2O_2, HCHO, nor •NO formation from these substrates was found to contribute significantly to NOS inactivation (Olken & Marletta, 1993). However, it remains to be determined whether the two electrophiles noted earlier, the iminium ion and the nitrone, participate in the inactivation of NOS during the processing of L-NMA and L-NHMA or whether other chemical entities could be involved (Scheme 2).

In this report, the mechanism-based inhibition by L-NMA was examined carefully, including determinations of turnover dependence, irreversibility, and the partition ratio of the inactivation. In addition, the stoichiometry of radiolabeling [^{14}C]-L-NMA and [^{3}H]-L-NMA was determined, as well as the stability of the radiolabeled enzyme to gel filtration and dialysis. UV−visible spectra of NOS were collected, which demonstrate that loss of the heme chromophore is associated with L-NMA inactivation. HPLC analysis of heme indicates that Fe-PPIX is lost during inactivation. Finally, a mechanistic scheme is presented to reconcile these findings with what is known of the mechanism of NOS and the manner of L-NMA and L-NHMA processing.

MATERIALS AND METHODS

General Methods. The citrulline assay utilizing [^{3}H]-L-NMA as substrate and the hemoglobin assay for NOS activity were carried out as described (Olken & Marletta, 1993). UV−visible spectroscopy was carried out on a Cary 3E spectrophotometer at 37 °C unless otherwise noted. Absolute spectra were normalized to zero at 700 nm and were corrected for dilution as required. Data were exported and processed using a Kaleidagraph 2.1 (Synergy Software, Reading, PA). Syntheses of [^{3}H]- and [^{14}C]-L-NMA were reported previously (Olken & Marletta, 1993). Dithionite-reduced CO difference spectra were obtained as described (White & Marletta, 1992). Incubations were treated with 20 units of catalase for 10 min at room temperature prior to the introduction of CO. This amount of catalase was found not to contribute to subsequent spectra and significantly reduced the P420 signal obtained from reactions with L-NMA. Protein concentration was determined with the Micro BCA kit (Pierce, Inc.) using bovine serum albumin as a standard. Liquid scintillation counting was carried out on a Beckman LS 1801 liquid scintillation counter. RAW 264.7 murine macrophages were grown, activated, and harvested as described (Tayeh & Marletta, 1989). NOS was purified as described (White & Marletta, 1992). H_2O_2 was measured as described (Olken & Marletta, 1993). Catalase (bovine liver, 44 units/μg), superoxide dismutase (SOD) (bovine erythrocyte), and dihydropteridine reductase (NADP$^+$, from sheep liver) were purchased from Sigma (St. Louis, MO). Dihydrofolate reductase (*Lactobacillus casei*) was the generous gift of Dr. Rowena Matthews (University of Michigan).

Turnover Dependence of Inactivation of NOS by L-NMA. Preincubation mixtures contained 4 μg of NOS (0.1 μM), 700 μM DTT, 100 μM NADPH, and 60 μM H_4B in 15 mM Hepes (pH 7.4) and 20% (v/v) glycerol in a total volume of 310 μL. Reactions were carried out in siliconized 16 × 100 mm test tubes cut to a height of approximately 5 cm. The tubes were sealed with rubber septa and preequilibrated under a gentle stream of argon for 10 min on ice with gentle swirling. The preincubation mixture was placed in a 37 °C shaking water bath for 3 min, and the reaction was initiated with 10 μL of a 3 mM solution of L-NMA (100 μM final concentration) introduced via a syringe. Aliquots (50 μL) were removed via syringe at times 0, 5, 10, 15, 20, and 25 min and assayed for NOS activity with oxyHb. Control incubations were carried out in an identical fashion, except that no argon was introduced into the head space during the 10 min preequilibration on ice.

Partition Ratio of [^{3}H]Citrulline Formation to NOS Inactivation for L-NMA. Incubations contained 0.1−1.0 μM NOS, 3 mM NADPH, 700 μM DTT, 60 μM H_4B, and 250 μM [^{3}H]- and [^{14}C]-L-NMA (final specific activities 0.73 and 0.19 mCi/mmol, respectively) in 15 mM Hepes (pH 7.4) and 10% (v/v) glycerol in a total volume of 1 mL. Reactions proceeded for 2 h at 37 °C, at which time NOS inactivation was complete. Aliquots of 800 μL were applied to 1 mL of Dowex AG 50W-X8 [Na^+], and [^{3}H]citrulline was measured by liquid scintillation counting.

Stoichiometry of Radiolabeling of NOS by [^{3}H]- and [^{14}C]-L-NMA. Incubations contained 150−300 μg of NOS (1−2 μM), 3 mM NADPH, 700 μM DTT, 60 μM H_4B, and 250 μM [^{3}H]- and [^{14}C]-L-NMA (final specific activities, 1.5 and 1.7 mCi/mmol, respectively) in 50 mM Hepes (pH 7.4) and 10% (v/v) glycerol in a total volume of 600−1000 μL. Reactions proceeded until inactivation was complete as determined via the oxyHb assay, typically 3 h. Aliquots of 500 μL were applied to 0.7 × 30 cm columns of Bio-Gel P-6 DG desalting gel (Bio-Rad, Hercules, CA) preequilibrated with 50 mM Hepes (pH 7.4). Fractions (1 mL) were collected and assayed for protein. Carbon-14 and tritium were determined from aliquots (250−600 μL) of the protein-containing fractions. Fractions collected before and after the protein-containing fractions were also counted to determine the background and to ensure baseline separation of protein and low molecular weight species.

Dialysis of Radiolabeled NOS. Radiolabeled NOS (250−500 μL) obtained via gel filtration (described earlier) was dialyzed against 90 mL of 50 mM Hepes (pH 7.4) using a Microdialyzer System 500 (Pierce, Rockford, IL). Gel filtration before dialysis was necessary because of the relatively low amounts of labeled NOS available for these experiments. In addition, dialysis permitted a distinction between tightly but reversibly bound and irreversibly bound radioactivity. Dialyses were carried out for 16 h at 4 °C. Following dialysis, samples were assayed for protein and radioactivity. Prior to dialysis, some samples were frozen and thawed twice (between −20 °C and 22 °C) or were heated to 70 °C for 5 min to denature the enzyme.

Effect of L-NMA Inactivation on Primary NOS Structure. Incubations contained 12 μg of NOS, 3 mM NADPH, 1 mM L-NMA or 1 mM L-arginine, 700 μM DTT, and 60 μM H_4B in 15 mM Hepes (pH 7.4) and 10% (v/v) glycerol in a total volume of 300 μL. Reactions proceeded for 2 h at 37 °C. Aliquots were removed at the end of the reactions for assay of NOS activity. Aliquots (approximately 5 μg) were also subjected to SDS−PAGE (10%) using a Mini-PROTEAN II cell (Bio-Rad, Hercules, CA). SDS−PAGE gels were visualized with silver stain.

Irreversibility of NOS Inactivation by L-NMA. Incubations contained 10−20 μg of NOS (0.15−0.3 μM), 1 mM L-NMA or 1 mM L-arginine (as a control), 1 mM NADPH, 700 μM DTT, and 60 μM H_4B in 15 mM Hepes (pH 7.4) and 10% (v/v) glycerol in a total volume of 500 μL. Aliquots of 50 μL were assayed for activity immediately after the reactions were initiated with NOS ($t = 0$) and following incubation at 37 °C for 45 min or 3 h. At this time, the remaining 400 μL of incubation mixture was applied to a 0.7 × 30 cm desalting column of Bio-Gel P-6 DG preequilibrated with 50 mM Hepes (pH 7.4) at 4 °C. Fractions (1 mL) were collected and assayed for protein and NOS activity. The specific activity of the L-NMA-treated samples was corrected

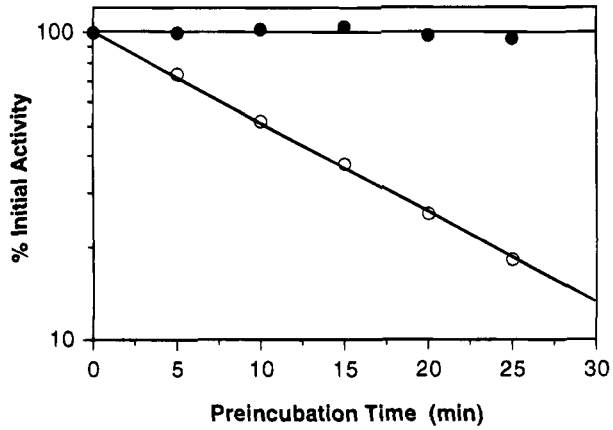


FIGURE 1: Turnover dependence of inactivation by L-NMA. Each preincubation mixture contained 4 μg of NOS, 700 μM DTT, 100 μM NADPH, and 60 μM H_4B in 15 mM Hepes (pH 7.4) and 20% (v/v) glycerol. Preincubations were equilibrated under an argon atmosphere (●) or under ambient air (○) for 10 min at 4 °C. The reactions were warmed to 37 °C and initiated with 100 μM L-NMA introduced via syringe. Aliquots were removed via syringe and assayed for NOS activity.

Table 1: Radiolabeling of NOS by [^{3}H]-L-NMA and [^{14}C]-L-NMA[a]

exp	post gel filtration[b] ([^{3}H]/[^{14}C])	dialysis: native[c] ([^{3}H]/[^{14}C])	dialysis: non-native ([^{3}H]/[^{14}C])
1A	0.11/0.51	ND	ND
1B	0.15/0.57	0.12/0.53	0.15/0.17[e]
1C	0.11/0.60	0.15/0.40	0.05/0.11[d]
2A	0.10/0.36	ND	ND
2B	0.09/0.36	0.07/0.36	ND
3	0.10/0.37	ND	0.07/0.11
4	0.12/0.36	ND	0.07/0.07
mean	0.11 ± 0.01/ 0.41 ± 0.10 (n = 4)	0.11 ± 0.04/ 0.43 ± 0.09 (n = 3)	0.08 ± 0.04/ 0.12 ± 0.04 (n = 4)

[a] Values are expressed as the number of equivalents of tritium or carbon-14 per NOS monomer. Each number refers to a different batch of radiolabeled NOS. Entries on the same line refer to manipulations carried out on the same aliquot of gel-filtered NOS. [b] Gel filtration of 500 μL aliquots of radiolabeled NOS was performed as described in Materials and Methods. [c] NOS dialyzed immediately following gel filtration or placed on ice during the manipulation of another portion of radiolabeled NOS. [d] Denaturing via heating to 70 °C for 5 min. [e] Denaturing via two freeze–thaw cycles.

for the loss (~10–50%) observed after gel filtration in the L-arginine controls.

Heme Analysis. Incubations for heme analysis contained 1–3 μM NOS, 2 mM L-NMA, and 3–6 mM NADPH in 50 mM Hepes (pH 7.4) and 10% (v/v) glycerol. Incubations also contained either 60 μM H_4B and 700 μM DTT (high DTT), 5 μM H_4B and 60 μM DTT (low DTT), or 100 μM H_4B (no DTT) and 0.5 unit of dihydrofolate reductase and 0.5 unit of dihydropteridine reductase. All incubations contained 100 units of catalase, and incubations without DTT also contained 150 units of SOD in a total volume of 600–1000 μL. Incubations proceeded for 3 h at 37 °C, at which time inactivation was typically about 75% complete. Oxygen depletion most likely limits the rate of turnover and, hence, inactivation when NOS is present in micromolar concentrations. HPLC analysis was carried out essentially as described (Kindt et al., 1992) with the following modifications. Samples were analyzed with a Vydac C18 (0.46 × 25 cm) reverse-phase column and eluted at 1 mL/min with solvent A (0.1% trifluoroacetic acid in H_2O) for 5 min, followed by a linear gradient from 0 to 75% solvent B (0.1% trifluoroacetic acid in CH_3CN) over 60 min. Absorbances were monitored at 400 and 220 nm with the use of a Beckman System Gold Model 121 solvent module and a Model 168 diode array detector.

RESULTS

Turnover Dependence of Inactivation by L-NMA. Incubations that contained NOS, NADPH, H_4B, and 100 μM L-NMA (K_I = 2.7 μM) were carried out under an argon atmosphere. These reaction mixtures demonstrated no loss of enzyme activity over 25 min (Figure 1). Identical incubations carried out under ambient atmosphere lost activity at the expected rate, giving a k_{inact} = 0.07 min^{-1} (Olken & Marletta, 1993). A control incubation with [^{3}H]-L-arginine under argon showed that turnover was inhibited by 96.4% relative to an identical incubation under ambient atmosphere.

Partition Ratio for L-NMA. Sets of five incubations containing NADPH, H_4B, [^{3}H]-L-NMA, and one of five concentrations of NOS were allowed to react until all of the NOS was inactivated. Plots of [^{3}H]citrulline formed vs NOS added were linear and intercepted the origin. A partition ratio of 108 ± 3 (n = 3) molecules of citrulline formed per NOS monomer inactivated was calculated from the slopes of these lines (not shown).

Radiolabeling of NOS by [^{3}H]- and [^{14}C]-L-NMA. NOS was reacted with NADPH, H_4B, and a substrate solution of [^{3}H]- and [^{14}C]-L-NMA. Following complete enzyme inactivation, gel filtration was performed to separate the protein and small molecules. As shown in Table 1, 0.11 ± 0.01 equiv of tritium and 0.41 ± 0.10 equiv of carbon-14 were bound per NOS monomer following gel filtration. Dialysis of aliquots of gel-filtered, radiolabeled NOS against buffer resulted in essentially no loss of either radiolabel. However, when gel-filtered, radiolabeled NOS was denatured prior to dialysis, 0.08 ± 0.04 equiv of tritium and 0.12 ± 0.04 equiv of carbon-14 per NOS monomer remained bound to the protein.

Irreversibility of NOS Inactivation by L-NMA. NOS that had been fully inactivated by L-NMA over 3 h regained no detectable activity following gel filtration. NOS that had been partially inactivated by preincubation with L-NMA (≤50% of the initial specific activity remained after the 45 min preincubation) was also subjected to gel filtration and reassayed for protein and activity. Of the specific activity lost during the preincubation (specific activity at t = 0 minus that at t = 45 min), 18 ± 8% (n = 3) was regained following gel filtration.

Effect of Inactivation on Primary NOS Structure. Incubations containing cofactors and either L-arginine (control) or L-NMA proceeded until NOS inactivation was complete. Aliquots of these incubations were subjected to SDS–PAGE, along with untreated NOS as a standard. Silver staining revealed only one band at M_r ~ 130 000 for each of these lanes (not shown). Therefore, no cleavage of the polypeptide chain of NOS accompanied inactivation by L-NMA.

Repetitive Scanning of Incubations with L-NMA. Addition of L-NMA to NOS resulted in a type I binding spectrum (Figure 2) similar to that observed previously with L-arginine and L-NHA (McMillan & Masters, 1993; Pufahl & Marletta,

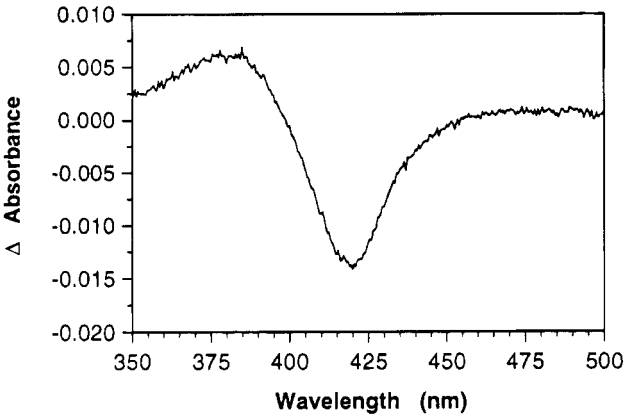


FIGURE 2: Type I differeence binding spectrum of L-NMA. L-NMA (100 μM) was added to oxidized NOS (2 μM), which contained 350 μM DTT and 30 μM H_4B in 50 mM Hepes (pH 7.4) and 20% (v/v) glycerol.

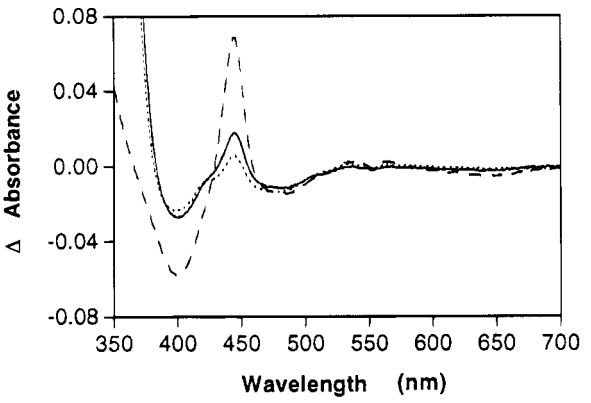


FIGURE 3: Reduced-CO difference spectra of NOS following inactivation with L-NMA. NOS (2 μM) was combined with 2 mM L-NMA, 3 mM NADPH, 350 μM DTT, and 30 μM H_4B (•••) or 40 units of catalase (—) and allowed to react for 3 h at 37 °C, after which time the reduced-CO difference spectra were obtained. NOS (2 μM) that did not undergo reaction was combined with 350 μM DTT and 30 μM H_4B (— —), and a reduced-CO difference spectrum was obtained. All samples were prepared in 50 mM Hepes (pH 7.4) and 20% (v/v) glycerol and contained identical concentrations of NOS.

1993). Repetitive scanning of incubations with L-NMA with or without catalase demonstrated a smooth loss of the Soret peak over time, which was not accompanied by new absorption bands and which paralleled the loss of NOS activity. Incubations that contained L-arginine lost less than 20% of the Soret peak height and NOS activity over several hours at 37 °C.

Loss of the P450 Chromophore Following Incubation with L-NMA. Figure 3 shows the reduced-CO difference spectra of incubations, which contained NOS, NADPH, H_4B, and L-NMA with or without catalase. The overall loss of P450 chromophore relative to the control (an identical concentration of NOS) indicated that catalase afforded some protection. Both incubations with L-NMA also appeared to contain significant concentrations of P420 as well. The L-NMA incubation that contained catalase retained 36% of the initial activity, while the L-NMA incubation that did not contain catalase retained only a trace of activity. Originally, identical incubations with L-arginine were used as the controls. However, these incubations rapidly formed a stable chromophore, which may be a heme−nitrosyl complex (A. R. Hurshman and M. A. Marletta, unpublished results). The

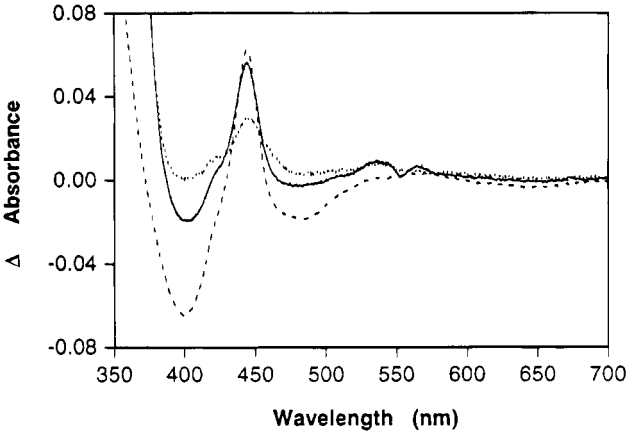


FIGURE 4: Reduced-CO difference spectra of NOS following incubation with no substrate. NOS (2 μM) was combined with 350 μM DTT, 30 μM H_4B, 3 mM NADPH, and 40 units of catalase (—) or no catalase (•••) in 50 mM Hepes (pH 7.4) and 20% (v/v) glycerol. Incubations proceeded for 3 h at 37 °C, and reduced-CO spectra were obtained. Also shown is an identical concentration of NOS (— —), that had not undergone reaction.

reduced-CO spectra could not be obtained from these samples without significant P420 formation. Therefore, identical concentrations of NOS that had been kept at 4 °C during the reactions were used as the control.

Effect of H_2O_2 Formation[2] on NOS Activity and the P450 Chromophore. Since the processing of L-NMA results in the formation of H_2O_2 via uncoupled NADPH oxidation (Olken & Marletta, 1993), the effect of H_2O_2 formation on NOS activity and the P450 chromophore was investigated. Figure 4 shows the reduced-CO difference spectra of reaction mixtures following a 3 h incubation of NOS, NADPH, and H_4B with or without catalase. In the presence of catalase, NOS retained almost all of the initial P450 chromophore and 80% of the initial activity. However, in the absence of catalase, NOS lost all detectable enzyme activity and most of the P450 chromophore.

Analysis of Heme. Incubations for heme analysis were carried out in the presence of high, low, or no DTT (see Materials and Methods) to maximize the probability of detecting any heme adducts formed. Thiols can cause degradation of heme or heme adducts in solution via redox cycling and oxidative destruction (Osawa et al., 1994). The three types of incubations yielded qualitatively similar results. Peak areas of Fe-PPIX from reaction mixtures of NOS and L-NMA were consistently smaller (typically 40−50%) than those obtained from equivalent amounts of native enzyme, indicating a reproducible loss of Fe-PPIX. As shown in Figure 5B, in addition to Fe-PPIX (retention time (t_r) = 47 min), three additional peaks (t_r = 6, 14, and 15 min) not present in the control (Figure 5A) were detected at 400 nm by reverse-phase high-performance liquid chromatography (RP-HPLC). These peaks appear to be due to $NADP^+$ and nucleotide breakdown products, as their UV−visible spectra (not shown) contain very high absorbances around 260 and

[2] Since H_2O_2 could initiate oxidative heme destruction or oxidative damage to a redox sensitive residue in the active site, an attempt was made to determine whether H_2O_2 *formation* was sufficient to inactivate NOS in the absence of H_2O_2 *accumulation* in solution. It is not known how the mechanisms of H_2O_2 formation in the absence of substrate compare to H_2O_2 formation during L-NMA processing, although the rates are similar (N. M. Olken and M. A. Marletta, unpublished results).

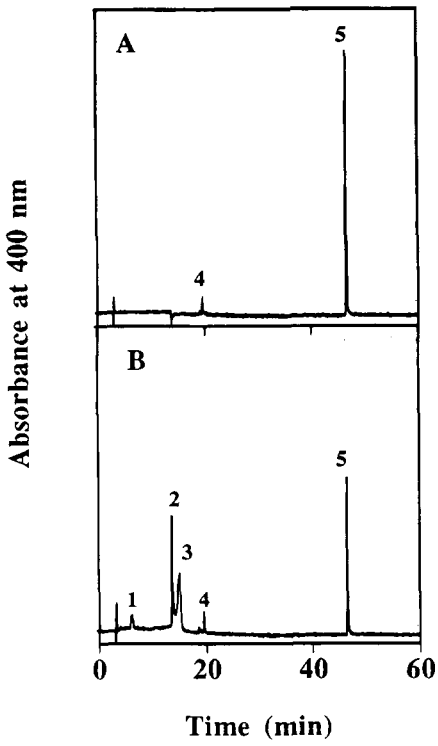


FIGURE 5: RP-HPLC analysis of heme from NOS. Panel A was obtained following the injection of 26 pmol of native NOS and demonstrates PPIX (t_r = 47 min) and flavin (t_r = 19 min). Panel B was obtained following the injection of 26 pmol of NOS inactivated with L-NMA. These chromatograms were obtained from reaction mixtures that contained no DTT, as described in Materials and Methods. In addition to Fe-PPIX (t_r = 47 min) and flavin (t_r = 19 min), three new peaks (numbered 1, 2, and 3 at t_r = 6, 14, and 15 min, respectively) were observed at 400 nm. Peaks 2 and 3 have UV−visible spectra similar to that of $NADP^+$, although the absorbance at 400 nm is unexplained.

280 nm. However, the relatively small absorbance at 400 nm is not consistent with $NADP^+$ and could indicate the presence of a small amount of heme adduct. Plasma desorption mass spectral analysis of the isolated peaks 2 and 3, as well as of the entire reaction mixture, was inconclusive given the small amounts of protein available.

DISCUSSION

In previous reports from our laboratory, several criteria for mechanism-based enzyme inactivation (Silverman, 1988) were shown to have been fulfilled for the inhibition of crude (Olken et al., 1991) and purified inducible macrophage NOS by L-NMA (Olken & Marletta, 1992, 1993). In particular, protection by substrate, saturability, and pseudo-first-order kinetics of inactivation (k_{inact} = 0.07 min^{-1}, K_I = 2.7 μM) were demonstrated. Feldman and co-workers have reported similar kinetic constants for the inactivation (Feldman et al., 1993). In this report, additional characterization of the inhibition has been carried out, including the determination of the partition ratio, the dependence of the inactivation on turnover, and the irreversible nature of the inactivation, as well as an initial probing of the mechanism of inactivation. The dependence of inactivation on turnover was demonstrated by the incubation of NOS with L-NMA and cofactors under an argon atmosphere. No inactivation was observed under argon at 37 °C over 25 min (Figure 1). The irreversible nature of the inactivation was demonstrated by gel filtration of NOS that had been completely or partially inactivated with L-NMA. Although small increases in the specific activity were evident following gel filtration of partially inactivated enzyme, in all cases the majority (>70%) of NOS activity that had been lost during preincubation with L-NMA was not restored. Gel filtration may have served to remove, from NOS, tightly but reversibly bound amino acid intermediates derived from L-NMA. The determination of the partition ratio was of particular interest, owing to the complexity of L-NMA processing. The amount of [^{3}H]-citrulline formed was found to be directly proportional to the concentration of NOS added, and a partition ratio of 108 ± 3 was determined from the slope of plots of the concentration of [^{3}H]citrulline formed vs the concentration of NOS added (not shown).

The mechanism of inactivation was investigated utilizing a variety of approaches, including inactivation with radiolabeled substrate followed by gel filtration and subsequent dialysis, UV−visible spectroscopy, and HPLC analysis of heme. The stoichiometry of radiolabeling of NOS by L-NMA was determined using a substrate solution composed of [^{14}C]- and [^{3}H]-L-NMA. This permitted the simultaneous tracking of both the amino acid backbone (via tritium) and the guanidino methyl group (via carbon-14). NOS was fully inactivated by radiolabeled L-NMA in the presence of excess NADPH and then subjected to gel filtration to remove radioactivity not bound to the protein. As shown in Table 1, the stoichiometry of tritium radiolabeling was found to be 0.11 ± 0.01 equiv per NOS monomer, and the stoichiometry of carbon-14 radiolabeling was found to be 0.41 ± 0.10 equiv per NOS monomer. Radiolabeled NOS isolated via gel filtration was then subjected to dialysis. NOS that had been placed on ice and then dialyzed against buffer lost essentially no radiolabel following overnight dialysis at 4 °C. However, denaturation of the radiolabeled NOS followed by dialysis resulted in significant losses of carbon-14 radiolabel (~0.3 equiv of carbon-14 per NOS monomer or 75% of the initial radiolabel), such that the resultant stoichiometry of tritium (amino acid backbone) to carbon-14 (guanidino methyl group) radiolabeling, although quite low, approached 1:1. Therefore, after complete inactivation of the enzyme with either tritium- or carbon-14-labeled NMA, the amount of labeling is substoichiometric. The differential labeling with ^{3}H and ^{14}C observed before denaturation and dialysis is consistent with there being multiple modes of inactivation. The low, but reproducible level of labeling following denaturation and dialysis with ^{3}H- and ^{14}C-labeled L-NMA is also consistent with covalent attachment of the entire L-NMA molecule to a small percentage of NOS subunits.

Scheme 2 illustrates a number of products potentially derived from the known processing of L-NMA by NOS that could serve as covalent enzyme inactivators, including the nitrone derived from L-NHMA (pathway 2) and an iminium ion derived from the initial *C*-hydroxylation product, the carbinolamine (pathway 1). Both could serve as Michael acceptors of an active site nucleophile. In addition, nitrones are also susceptible to radical addition reactions. The finding of a higher level of radiolabeling by [^{14}C]-L-NMA relative to [^{3}H]-L-NMA prior to enzyme denaturation suggests that NOS is modified by a fragment of L-NMA that includes the guanidino [^{14}C]methyl carbon but that does not include the ^{3}H-containing amino acid backbone. Nitrosomethane radical cation formation (pathway 3) from L-NHMA (discussed later)

could account for this part of the radiolabeling data. The reversibility of the carbon-14 radiolabel upon enzyme denaturation has two likely explanations. Either a cofactor is modified that is subsequently released from NOS upon denaturation, or a hydrolytically sensitive covalent bond is formed that is initially shielded from H_2O in the active site but is exposed and then hydrolyzed upon enzyme denaturation.

UV−visible spectroscopy was used to assess spectral changes in the heme absorbance that could indicate the mechanism of NOS inhibition. In an initial series of experiments, it was found that incubations of NOS, cofactors, and L-NMA lost the Soret absorbance at ~394 nm at a much faster rate than similar incubations carried out with L-arginine (not shown), without the formation of any new absorbances suggestive of a metabolic inhibitory complex (Franklin, 1991). As shown in Figure 3, incubations of NOS with cofactors and L-NMA in the absence or presence of catalase each resulted in a profound loss of P450 chromophore. Catalase did protect the P450 chromophore to a small degree over the course of a prolonged (3 h) incubation with L-NMA. These results indicate that the *accumulation* of H_2O_2 in solution may contribute to the overall rate of inactivation of NOS and loss of P450 chromophore by L-NMA under conditions where the H_2O_2 concentration can be relatively high (approaching 1 mM). However, when H_2O_2 accumulation is much lower, such as occurs during short incubations (≤30 min) of relatively dilute concentrations of NOS (≤0.1 μM) with L-NMA, no protection was observed in the presence of catalase (Olken & Marletta, 1993). As shown in Figure 4, the *formation*[2] of H_2O_2 in the absence of substrate did not, in and of itself, lead to NOS inactivation and P450 chromophore loss when catalase was present to prevent the *accumulation* of H_2O_2 in solution. In the absence of catalase, most of the P450 chromophore and all NOS activity were lost. This vulnerability of NOS to H_2O_2 in solution, particularly in the absence of substrate, has been observed for other P450 enzymes and results from oxidative heme destruction (Guengerich, 1978; Schaefer et al., 1985).

RP-HPLC analysis of heme from native NOS revealed only Fe-PPIX (Figure 5A, $t_r = 47$ min). L-NMA-inactivated NOS showed a loss in PPIX, as well as three additional peaks detectable at 400 nm, the two most prominent of which appear to be due to $NADP^+$ or nucleotide breakdown products (Figure 5B). Loss of Fe-PPIX could result from heme modification or destruction by a reactive intermediate derived from L-NMA. However, heme modification probably would not occur by pathways 1 or 2 in Scheme 2, since the pyrrole nitrogens of PPIX are not nucleophilic in P450 enzymes (Ortiz de Montellano, 1986). Pathway 3 of Scheme 2 illustrates the formation of nitrosomethane radical cation, a highly reactive species that may be derived from the methyl-bearing guanidino nitrogen of L-NHMA (Feldman et al., 1993). The formation of this molecule would be consistent with the observed loss of Fe-PPIX (via either heme modification or oxidative destruction), the higher level of radiolabeling by [^{14}C]-L-NMA relative to that by [^{3}H]-L-NMA, and the more rapid inactivation of NOS by L-NHMA [$k_{inact} = 0.16$ min^{-1} (Pufahl et al., 1992)] relative to that by L-NMA [$k_{inact} = 0.07$ min^{-1} (Olken & Marletta, 1993)]. The solution chemistry of nitrosomethane radical cation is not known, although in the gas phase it appears to fragment into a methyl radical and a nitronium ion (Hop et al., 1991).

The degree of spectroscopic heme loss of NOS mediated by L-NMA ordinarily would indicate without ambiguity that the primary mechanism of irreversible inactivation of a P450 hemoprotein occurred via heme modification or destruction. However, the inducible NOS is active as a homodimer and has been reported to reversibly dissociate into inactive monomers and free PPIX (Baek et al., 1993). This finding suggests that the loss of heme chromophore could be the result of protein modification, which then results in the dissociaton of dimer into inactive monomers and free heme. However, L-NMA-inactivated NOS was found to be primarily dimeric by native PAGE, suggesting that dimer dissociation contributes little to the observed loss of heme chromophore.[3] This finding is corroborated by the RP-HPLC results that would have detected free PPIX as well as that which was protein bound. Therefore, the loss of heme chromophore by UV−visible spectroscopy, taken together with the RP-HPLC analysis of heme, indicates that heme loss (via oxidative destruction or modification) is a contributing pathway to the inactivation of NOS by L-NMA.

These studies were designed to determine whether a major heme adduct could be detected that would account for the heme loss observed in the spectroscopy experiments. None was found, which suggests that the product is unstable, that multiple products are formed in smaller amounts, or that destruction of the heme occurs, leading to breakdown products that were undetected by the methods employed. Covalent modification of the protein backbone of cytochrome P450 hemoproteins is a relatively unusual event as the primary mechanism of inactivation, presumably because the heme is perfectly positioned to intercept reactive intermediates formed in an otherwise hydrophobic active site. However, the active site of NOS most likely contains a number of polar or charged residues involved in L-arginine binding, which may be tempting targets for electrophilic intermediates formed during the processing of L-arginine analogs. The study of L-NMA and other simple N^G-substituted L-arginine analogs provides a novel perspective on the active site chemistry of this unusual family of P450 enzymes, which is now known to have an integral role in mammalian physiology and pathophysiology.

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REFERENCES

Akhtar, M., Calder, M. R., Corina, D. L., & Wright, J. N. (1982) *Biochem. J. 201*, 569−580.

Baek, K. W., Thiel, B. A., Lucas, S., & Stuehr, D. J. (1993) *J. Biol. Chem. 268*, 21120−21129.

Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., & Snyder, S. H. (1991) *Nature (London) 351*, 714−718.

Feldman, P. L., Griffith, O. W., Hong, H., & Stuehr, D. J. (1993) *J. Med. Chem. 36*, 491−496.

Franklin, M. R. (1991) *Methods Enzymol. 206*, 559−573.

Guengerich, F. P. (1978) *Biochemistry 17*, 3633−3639.

[3] N. M. Olken and M. A. Marletta, unpublished results.

Hevel, J. M., & Marletta, M. A. (1992) *Biochemistry 31*, 7160–7165.
Hevel, J. M., White, K. A., & Marletta, M. A. (1991) *J. Biol. Chem. 266*, 22789–22791.
Hop, C. E. C. A., Chen, H., Ruttink, P. J. A., & Holmes, J. L. (1991) *Org. Mass Spectrom. 26*, 679–687.
Kindt, J. T., Woods, A., Martin, B. M., Cotter, R. J., & Osawa, Y. (1992) *J. Biol. Chem. 267*, 8739–43.
Marletta, M. A. (1993) *J. Biol. Chem. 268*, 12231–12234.
Marletta, M. A., Yoon, P. S., Iyengar, R., Leaf, C. D., & Wishnok, J. S. (1988) *Biochemistry 27*, 8706–8711.
Mayer, B., John, M., Heinzel, B., Werner, E. R., Wachter, H., Schultz, G., & Bohme, E. (1991) *FEBS Lett. 288*, 187–191.
McMillan, K., & Masters, B. S. S. (1993) *Biochemistry 32*, 9875–9880.
McMillan, K., Bredt, D. S., Hirsch, D. J., Snyder, S. H., Clark, J. E., & Masters, B. S. S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11141–11145.
Nathan, C. (1992) *FASEB J. 6*, 3051–3064.
Nelson, D. R., Kamataki, T., Waxman, D. J., Guengerich, F. P., Estabrook, R. W., Feyereisen, R., Gonzalez, F. J., Coon, M. J., Gunsalus, I. C., Gotoh, O., Okuda, K., & Nebert, D. W. (1993) *DNA Cell Biol. 12*, 1–51.
Olken, N. M., & Marletta, M. A. (1992) in *The biology of nitric oxide. 2 Enzymology, biochemistry and immunology* (Moncada, S., Marletta, M. A., Hibbs, J. B., Jr., & Higgs, E. A., Eds.) pp 24–28, Portland Press, London.
Olken, N. M., & Marletta, M. A. (1993) *Biochemistry 32*, 9677–9685.
Olken, N. M., Rusche, K. M., Richards, M. K., & Marletta, M. A. (1991) *Biochem. Biophys. Res. Commun. 177*, 828–833.
Ortiz de Montellano, P. R. (1986) in *Cytochrome P-450 structure, mechanism, and biochemistry* (Ortiz de Montellano, P. R., Ed.) pp 273–315, Plenum Press, New York.
Osawa, Y., Fellows, C. S., Meyer, C. A., Woods, A., Castoro, J. A., Cotter, R. J., Wilkins, C. L., & Highet, R. J. (1994) *J. Biol. Chem. 269*, 15481–15487.
Pufahl, R. A., & Marletta, M. A. (1993) *Biochem. Biophys. Res. Commun. 193*, 963–970.
Pufahl, R. A., Nanjappan, P. G., Woodard, R. W., & Marletta, M. A. (1992) *Biochemistry 31*, 6822–6828.
Schaefer, W. H., Harris, T. M., & Guengerich, F. P. (1985) *Biochemistry 24*, 3254–63.
Schmidt, H. H. H. W., Smith, R. M., Nakane, M., & Murad, F. (1992) *Biochemistry 31*, 3243–3255.
Silverman, R. B. (1988) in *Mechanism-based enzyme inactivation: chemistry and enzymology*, pp 3–30, CRC Press, Boca Raton, FL.
Stuehr, D. J., & Ikeda-Saito, M. (1992) *J. Biol. Chem. 267*, 20547–20550.
Stuehr, D. J., Cho, H. J., Kwon, N. S., Weise, M. F., & Nathan, C. F. (1991a) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7773–7777.
Stuehr, D. J., Kwon, N. S., Nathan, C. F., Griffith, O. W., Feldman, P. L., & Wiseman, J. (1991b) *J. Biol. Chem. 266*, 6259–6263.
Tayeh, M. A., & Marletta, M. A. (1989) *J. Biol. Chem. 264*, 19654–19658.
Wang, J., Stuehr, D. J., Ikeda-Saito, M., & Rousseau, D. L. (1993) *J. Biol. Chem. 268*, 22255–22258.
White, K. A., & Marletta, M. A. (1992) *Biochemistry 31*, 6627–6631.