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Mechanism of Inactivation of Inducible Nitric Oxide Synthase by Amidines. Irreversible Enzyme Inactivation without Inactivator Modification

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Abstract: Nitric oxide synthases (NOS) are hemoproteins that catalyze the reaction of L-arginine to L-citrulline and nitric oxide. *N*-(3-(Aminomethyl)benzyl)acetamidine (1400W) was reported to be a slow, tight-binding, and highly selective inhibitor of iNOS in vitro and in vivo. Previous mechanistic studies reported that 1400W was recovered quantitatively after iNOS fully lost its activity and modification to iNOS was not detected. Here, it is shown that 1400W is a time-, concentration-, and NADPH-dependent irreversible inactivator of iNOS. HPLC-electrospray mass spectrometric analysis of the incubation mixture of iNOS with 1400W shows both loss of heme cofactor and formation of biliverdin, as was previously observed for iNOS inactivation by another amidine-containing compound, N^5-(1-iminoethyl)-L-ornithine (L-NIO). The amount of biliverdin produced corresponds to the amount of heme lost by 1400W inactivation of iNOS. A convenient MS/MS−HPLC methodology was developed to identify the trace amount of biliverdin produced by inactivation of iNOS with either 1400W or L-NIO to be biliverdin IXα out of the four possible regioisomers. Two mechanisms were previously proposed for iNOS inactivation by L-NIO: (1) uncoupling of the heme peroxide intermediate, leading to destruction of the heme to biliverdin; (2) abstraction of a hydrogen atom from the amidine methyl group followed by attachment to the heme cofactor, which causes the enzyme to catalyze the heme oxygenase reaction. The second mechanistic proposal was ruled out by inactivation of iNOS with d_3-1400W, which produced no d_2-1400W. Detection of carbon monoxide as one of the heme-degradation products further excludes the covalent heme adduct mechanism. On the basis of these results, a third mechanism is proposed in which the amidine inactivators of iNOS bind as does substrate L-arginine, but because of the amidine methyl group, the heme peroxy intermediate cannot be protonated, thereby preventing its conversion to the heme oxo intermediate. This leads to a change in the enzyme mechanism to one that resembles that of heme oxygenase, an enzyme known to convert heme to biliverdin IXα. This appears to be the first example of a compound that causes irreversible inactivation of an enzyme without itself becoming modified in any way.

Introduction

Nitric oxide, which is produced by the enzyme nitric oxide synthase (NOS, EC 1.14.13.39), is an important cell-signaling agent.[1] An explosion of research in this area has demonstrated that nitric oxide fulfills a large range of biological functions as both a messenger and a cytotoxic factor.[2] Because of the harmful effects of excess NO, inhibition of NO biosynthesis has been an important approach to the design of new potential drugs to treat those diseases caused by NO overproduction. However, NOS inhibition may also be detrimental to the essential functions of nitric oxide. This dilemma can be alleviated by selective inhibition of the three NOS isoforms, neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS), which have different functions.

NOS converts L-arginine (**1**) and O_2 to L-citrulline (**2**) and NO (**3**) with concomitant oxidation of NADPH (Scheme 1). The three isoforms of nitric oxide synthase are homodimeric hemoproteins incorporating several domains in a single polypeptide: (a) a heme domain that also binds a tetrahydrobiopterin cofactor and is the site of oxidation of L-arginine to nitric oxide and L-citrulline,[3] (b) a P450 reductase-like domain that has binding sites for FMN, FAD, and NADPH and is responsible for providing electrons to the heme domain,[4] and (c) a connecting peptide between the heme and flavin domains that binds calmodulin in a Ca^{2+}-dependent manner for the endothelial

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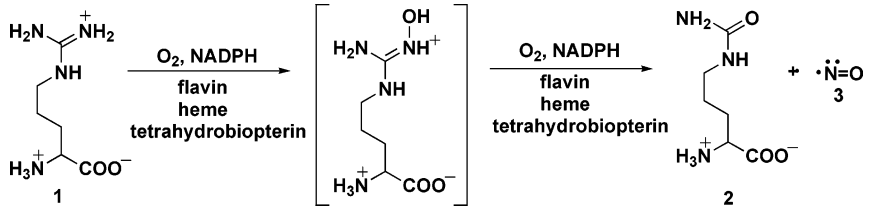
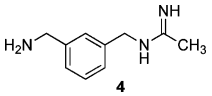
Scheme 1

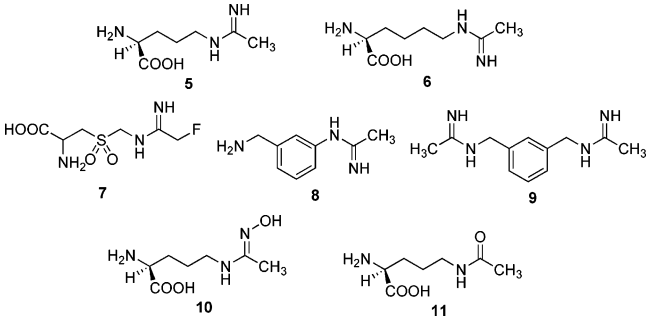


(eNOS) and neuronal (nNOS) forms and an essentially Ca^{2+}-independent manner in the inducible enzyme (iNOS) because it binds Ca^{2+} so tightly it is insensitive to changes in cellular Ca^{2+} levels.[5]

Garvey and co-workers reported 1400W (**4**) to be a slow, tight-binding, and highly selective

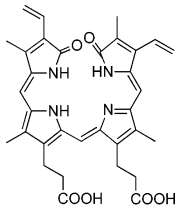


inhibitor of iNOS in vitro and in vivo.[6] 1400W was the most selective inhibitor of purified human iNOS reported to date (5000- and 2000-fold more potent against purified human iNOS than eNOS and nNOS, respectively). Inhibition of human iNOS by 1400W did not reverse upon dilution; consequently, 1400W was initially concluded to be either a slowly reversible inhibitor or an irreversible inactivator. However, additional mechanistic studies also established that 1400W was quantitatively recovered, and no modification of iNOS could be detected, supporting reversible inhibition. 1400W is one of several reported amidine-containing inhibitors of NOS, including N^5-(1-iminoethyl)-L-ornithine (L-NIO, **5**),[7] a natural product[8] known to be a substrate and an inactivator of

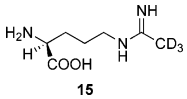


iNOS, N^5-(1-iminoethyl)-L-lysine (L-NIL; homo- L-NIO, **6**), which inactivates iNOS by modifying its heme cofactor and destabilizing the iNOS homodimer into inactive monomers,[9,10] 2-amino-6-(1-imino-2-fluoroethylamino)-4,4-dioxo-4-thiohexanoic acid (**7**),[11] *N*-(3-(aminomethyl)phenyl)acetamidine (5320W, **8**), which differs from 1400W by only one methylene unit but is nNOS selective,[12] and *N*,*N*′-(1,3-benzyl)bisamidine (**9**).[6,12]

The recovery of 1400W with slowly reversible or irreversible iNOS activity is reminiscent of the mechanistic behavior of L-NIO[7] (**5**). L-NIO was both a substrate and inactivator for iNOS. After incubation with iNOS, most of the L-NIO was recovered; a minor part was converted to N^G-hydroxy-L-NIO (**10**) and L-acetylornithine (**11**), which were irrelevant to the L-NIO inactivation mechanism.[7] Complete inactivation of iNOS by L-NIO occurred concomitant with loss of heme cofactor from the homodimer and formation of biliverdin (**12**).



Biliverdin is the major product of the heme-degradation reaction catalyzed by the enzyme heme oxygenase;[13] however, there was no heme oxygenase in the iNOS preparation. Two mechanisms to rationalize the formation of biliverdin that were proposed earlier[7] were (1) L-NIO attaches to the meso position of the heme (**13**), which changes the chemistry to that resembling heme oxygenase, producing biliverdin (**12**) via verdoheme (**14**) (Scheme 2) and (2) the lack of a proton source in the active site prevents the conversion of peroxoheme to the iron oxo species, releasing excess peroxide, leading to α-*meso*-hydroxyheme (Scheme 3). The first mechanism was based on a variety of experiments using [^{14}C]- and [^{2}H]-labeled L-NIO, especially the small deuterium isotope effect observed on inactivation by trideuteriomethyl-L-NIO (**15**), suggesting a possible C−H bond



cleavage during inactivation.[7] The intriguing aspect of the first mechanism is that it accounts for little loss of inactivator because the inactivator is regenerated by spontaneous decarboxylation of the proposed product. The first mechanism also is supported by the observation that α-*meso*-methylheme is a substrate for heme oxygenase and is converted to biliverdin;[14]

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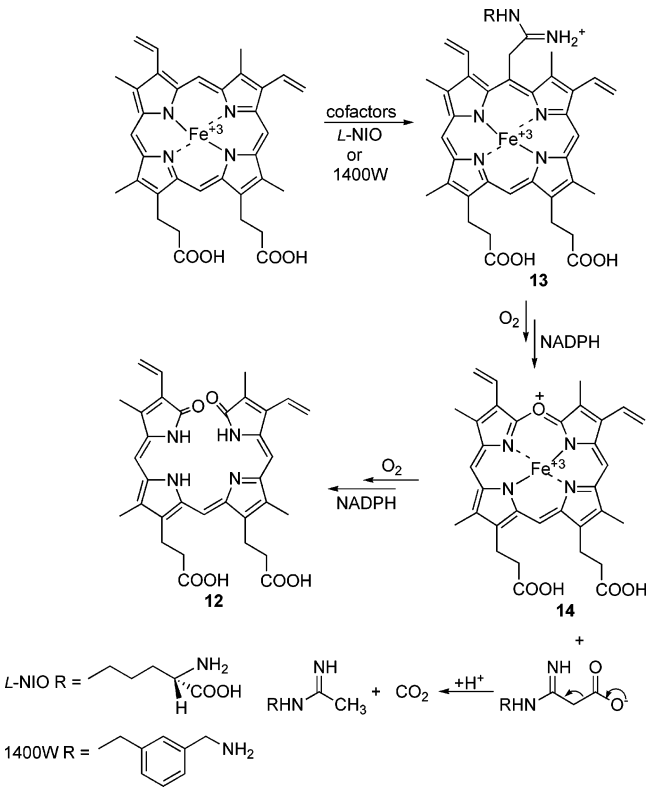
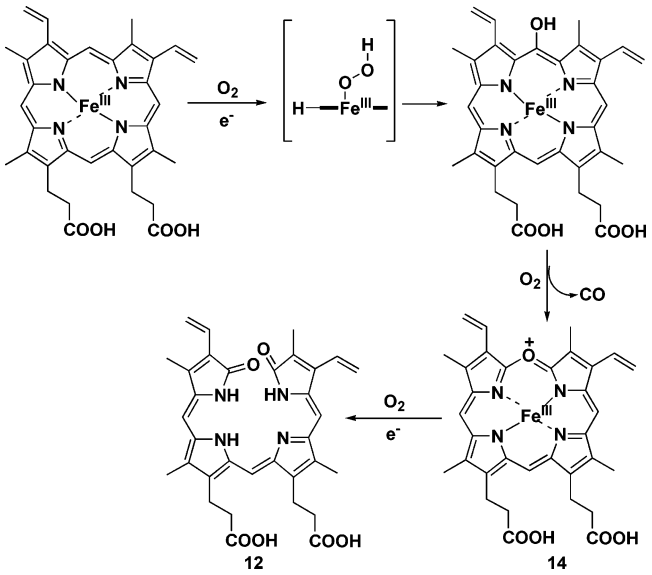
Scheme 2



Scheme 3



presumably, **13** (Scheme 2) could function the same as α-*meso*-methylheme.

In the second mechanism, the heme cofactor of NOS is hydroxylated at the α-meso-position to form α-*meso*-hydroxyheme, which is then decomposed to biliverdin and CO. The second mechanism initially seemed less appealing because it does not appear to rationalize the deuterium isotope effect on inactivation.

Here, we present experiments to demonstrate that 1400W and L-NIO apparently inactivate iNOS by the same mechanism, that the biliverdin that is formed during inactivation is one regioisomer, namely, biliverdin IXα, the same isomer that is produced by heme oxygenase-catalyzed oxidation of heme, and that the first proposed mechanism above is not reasonable but a heme oxygenase-like mechanism is reasonable.

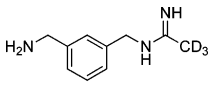
Table 1. K_I and k_{inact} Values for 1400W and L-NIO with iNOS

	1400W	L-NIO
K_I (μM)	23.8 ± 1.04	13.7 ± 1.60
k_{inact} (min^{-1})	2.40 ± 0.28	0.073 ± 0.003
k_{inact}/K_I (μM^{-1} min^{-1})	0.10 ± 0.01	0.0053 ± 0.0007

Results

Inactivation of iNOS by 1400W. As in L-NIO, 1400W was a time-, concentration-, and NADPH-dependent inactivator of iNOS; 1400W is a more efficient inactivator than L-NIO (Table 1).

Kinetic Isotope Effect on Inactivation of iNOS by *N*-(3-(Aminomethyl)benzyl)-[$^2H_3$]-acetamidine (12). *N*-(3-(Aminomethyl)benzyl)-[$^2H_3$]-acetamidine (**16**) was synthesized from CD_3CN via 2-[$^2H_3$]-acetimidic acid ethyl ester (Scheme 4).



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Inactivation by **16** shows a kinetic isotope effect on $^Hk_{inact}/^Dk_{inact}$ of 1.33 ± 0.18 and on $^H(k_{inact}/K_I)/^D(k_{inact}/K_I)$ of 1.39 ± 0.20 (Table 2 and Supporting Information).

Heme Loss during Inactivation by 1400W. iNOS incubation with 1400W and all necessary cofactors was subjected to high-performance liquid chromatography (HPLC) analysis after three hours (Figure 1). The heme cofactor appears to be involved in the inactivation event because the peak area for the heme cofactor decreased with concomitant loss of enzyme activity. A control solution made by either omitting NADPH or 1400W maintained most of its activity. Inclusion of catalase in the inactivation mixtures decreased the amount of heme loss in the control solutions but did not diminish significant loss of heme (40%) in the solution containing both 1400W and NADPH.

Formation of a New Peak after Inactivation. The HPLC trace after iNOS inactivation also reveals a new NADPH- and 1400W-dependent peak at 6.5 min, which is consistent with the retention time for commercial biliverdin IXα (Figure 1). The formation of the new peak also occurs in a time-dependent manner (data not shown).

Characterization of the New Peak from 1400W Inactivation. LC-electrospray mass spectrometry was used to compare the control (no 1400W), inactivation (with 1400W), and standards of commercial biliverdin IXα and hemin (ferriprotoporphyrin IX chloride). The mass spectra of the peaks in the LC-MS traces (see Supporting Information) at 14.4 and 16.4 min from the inactivation mixture are shown in Figure 2. The *m*/*z* values for the 14.4 min peak and 16.4 min peak are 583 (Figure 2A) and 616 (Figure 2B), respectively. MS/MS of the 14.4 min peak in the inactivation trace gave a spectrum (Figure 3A), which is nearly identical with that of the commercial biliverdin IXα (Figure 3B). Both MS/MS spectra show a base peak with *m*/*z* of 297.3. The 297.3 peak can be rationalized by a "half-half" fragmentation pattern that is not consistent with that expected for the β- and δ-isomers of biliverdin (Figure 4).

Identification of the Biliverdin Regioisomer Produced from Inactivation of iNOS using MS/MS Analysis.

Scheme 4

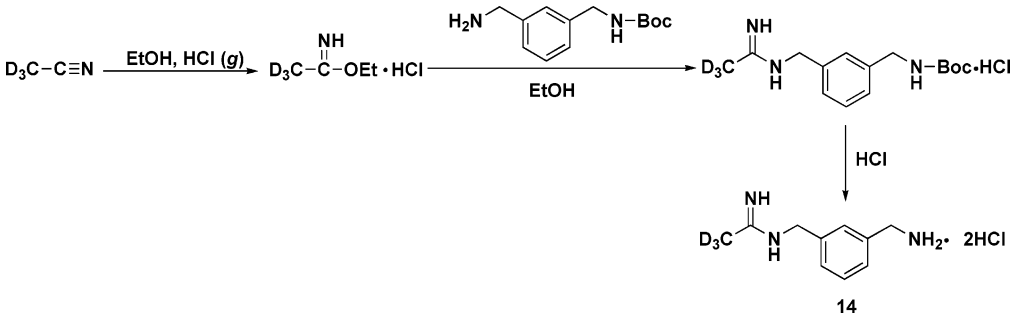

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Table 2. Kinetic Isotope Effect on INOS Inactivation by 1400W and d_3-1400W

	1400W	d_3-1400W	H/D
k_{inact} (min^{-1})	2.40 ± 0.28	1.80 ± 0.12	1.33 ± 0.18
K_I (μM)	23.8 ± 1.04	24.8 ± 0.62	0.96 ± 0.05
k_{inact}/K_I (μM^{-1} min^{-1})	0.101 ± 0.012	0.073 ± 0.005	1.39 ± 0.20

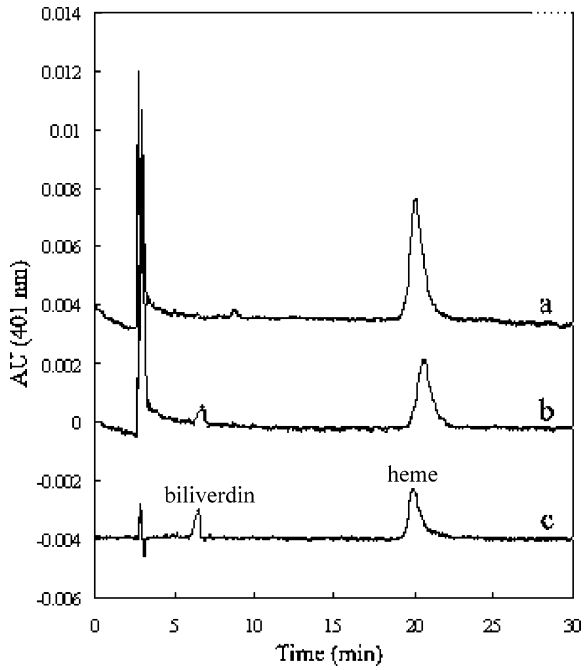

Figure 1. HPLC of heme loss and formation of a new peak (6 min) after iNOS inactivation by 1400W. (a) Control (without 1400W); (b) inactivation (with 1400W); (c) biliverdin and heme standards.

The four biliverdin IX regioisomers were synthesized[24,38] and subjected to MS/MS analysis under conditions used in the characterization of the new peak during iNOS inactivation. The MS/MS analyses (Figure 5) show that the α-, β-, γ-, and δ-isomers have very different fragmentation patterns, and the β-, γ-, and δ-isomers do not give a fragmentation with m/z of 297.2. Therefore, the unknown biliverdin from iNOS inactivation by both 1400W and L-NIO is biliverdin IXα.

Identification of the Biliverdin Regioisomer Produced from Inactivation of iNOS by HPLC Analysis. The four biliverdin IX regioisomers were separated by reversed-phase HPLC with 370 nm wavelength detection in the order of α, $\beta+\delta$, γ with retention times of 24.7, 27.4, and 35.6 min, respectively (Figure 6). The biliverdin peaks in the HPLC traces of the incubation of iNOS inactivation by 1400W and L-NIO have the same retention time as that of the α-isomer reference compound (the tiny peak at 29.3 min is the heme cofactor).

Quantification of the Formation of Biliverdin and Loss of Heme. HPLC traces of biliverdin IXα and hemin standards at different concentrations were recorded and peak areas were calculated to make calibration curves (see Supporting Information) to quantify the biliverdin formed from heme degradation by iNOS inactivation with 1400W and L-NIO. The ratio of biliverdin to heme loss was calculated to be 0.90 ± 0.04 for iNOS inactivation by 1400W and 0.92 ± 0.06 by L-NIO.

Determination of Deuterium Loss During Inactivation of iNOS by d_3-1400W. To determine if a C−H bond of the amidine methyl group is broken during iNOS inactivation, as proposed in the alkylated heme mechanism (Scheme 2), d_3-1400W was incubated with iNOS. After the incubation mixture fully lost its activity, the inactivator (heme:inactivator = 1:5) was separated from the enzyme by ultrafiltration and then derivatized with OPA-DTT reagent (see Supporting Information) to an OPA-DTT-d_3-1400W adduct ($M+H^+$ = 433.6).[15] By comparing the calculated peak areas of the adducts in the HPLC traces of inactivation (containing enzyme) and control (no enzyme), the total amount of inactivator was fully recovered. LC-APCI mass spectrometry was used to analyze the fully recovered inactivator from iNOS inactivation in its adduct form ($M+H^+$ = 433.6). The mass spectrum showed no peak with a mass value of 432.6, which would have corresponded to OPA-DTT- d_2-1400W plus a proton (see Supporting Information).

Detection of CO as One of the Heme Metabolites. Freshly prepared ferrous deoxymyoglobin was used as the trapping reagent to detect the possible formation of CO as an oxidation product of heme during inactivation of iNOS by L-NIO and 1400W. iNOS inactivation by L-NIO and 1400W produced a shift of the Soret maximum from 430 nm (ferrous deoxymyoglobin) to 418 nm (Figure 7), which indicates complete complexation of CO with the ferrous ion.[14] The positive control (replacing the incubation mixture with CO-saturated buffer) and the negative control (without inactivators) confirmed this result.

Discussion

Our earlier mechanistic studies demonstrated that L-NIO inactivates iNOS by degrading its heme cofactor into biliverdin but with no apparent modification to L-NIO itself.[7] Likewise, the highly selective, non-amino acid-based, amidine-containing iNOS inhibitor, 1400W, was reported to be a slowly reversible inhibitor or irreversible inactivator, but no chemical modification of the enzyme or of 1400W was detected after iNOS inactiva-

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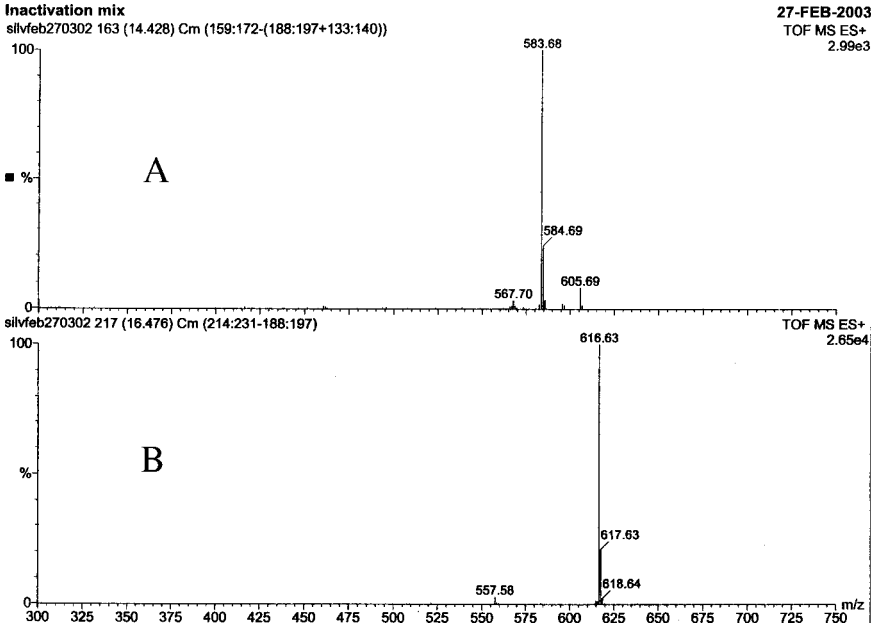

Figure 2. Mass spectra of the 14.4 min (6.5 min in Figure 1b) peak A and the 16.4 min (20.5 min in Figure 1b) peak B in the inactivation LC trace.

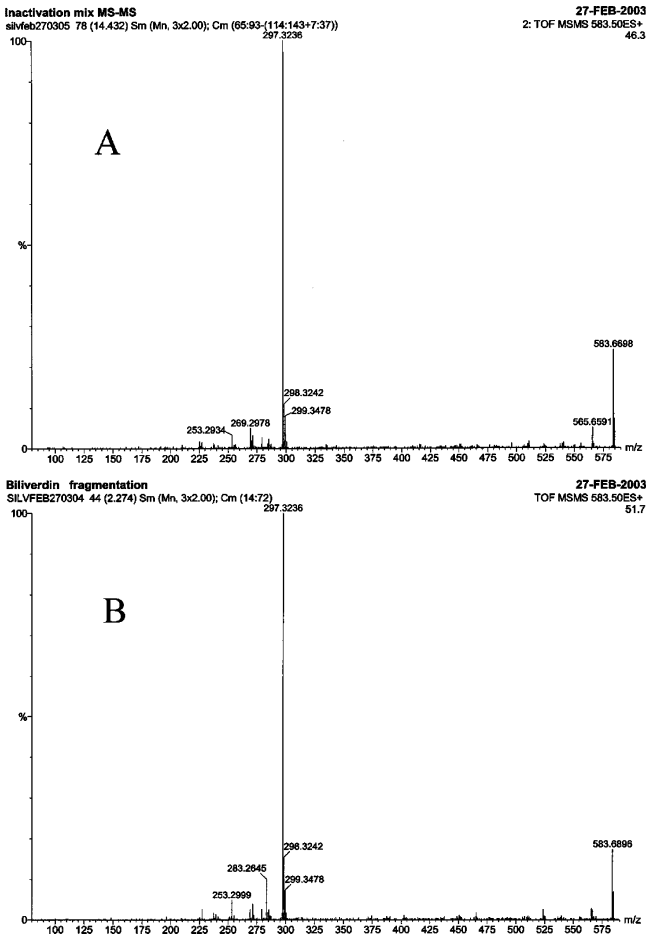

Figure 3. (A) MS/MS of the *m*/*z* 583 peak in Figure 2A. (B) MS/MS of biliverdin IXα standard.

tion.[6] A comparison of the structures of 1400W and L-NIO suggests that they could bind to and inhibit iNOS similarly. However, our results with L-NIO and those of Garvey and coworkers[6] with 1400W appear to indicate that the mechanisms of action of these two inhibitors are different. To determine if both amidine-containing inhibitors have a common mechanism of action and to characterize the enzyme modification product further, we investigated the inhibition of iNOS by 1400W.

1400W was a time-, concentration-, and NADPH-dependent inhibitor of iNOS. In fact, the k_{inact}/K_I value for 1400W is about 20 times larger than that for L-NIO (Table 1). Also, as was found earlier for L-NIO, *N*-(3-(aminomethyl)benzyl)-2-[$^2H_3$]-acetamidine (d_3-1400W) inactivated iNOS with a kinetic deuterium isotope effect on k_{inact}/K_I of 1.39 ± 0.2 (Table 2). Initially, it was not clear if this was a small primary kinetic isotope effect or a large secondary kinetic isotope effect (see below). L-NIO caused inactivation of iNOS with concomitant conversion of its heme to biliverdin (**12**). HPLC analysis was used to detect possible metabolites of 1400W-inactivated iNOS. Unlike that reported previously,[6] the heme cofactor of iNOS was diminished with a concomitant decrease in enzyme activity, suggesting that the heme cofactor was involved in the inactivation event (Figure 1). A new time-dependent, NADPH-dependent, and 1400W-dependent small peak was found by HPLC with the same retention time as that of a biliverdin standard (Figure 1), and LC-MS analysis confirmed the new peak to be biliverdin (Figures 2 and 3). On the basis of HPLC peak areas, it was calculated that 0.90 ± 0.04 equivalent of biliverdin was formed per 1.00 equivalent of heme loss during iNOS inactivation by 1400W, and 0.92 ± 0.06 equivalent of biliverdin was formed per 1.00 equivalent of heme loss during iNOS inactivation by L-NIO (calibration curves were made using commercial hemin and biliverdin IXα after the identification of the unknown biliverdin). These results support 1400W as an iNOS inactivator, which acts very similarly to that of L-NIO. Previously, we found that N^G-allyl-L-arginine inactivates NOS by allylation and reduction of the heme,[16] indicating that nonamidine-containing inactivators do not modify the heme cofactor by conversion to biliverdin. This demonstrates that amidines inactivate NOS by a unique mechanism.

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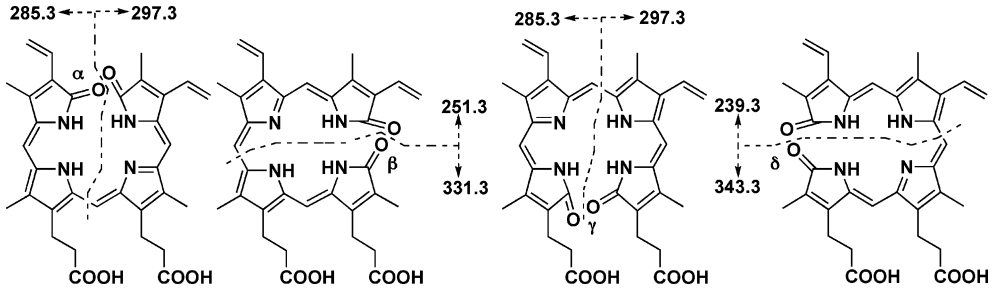

Figure 4. Expected *m*/*z* values for splitting of biliverdin IX α, *β*, *γ*, and *δ*.

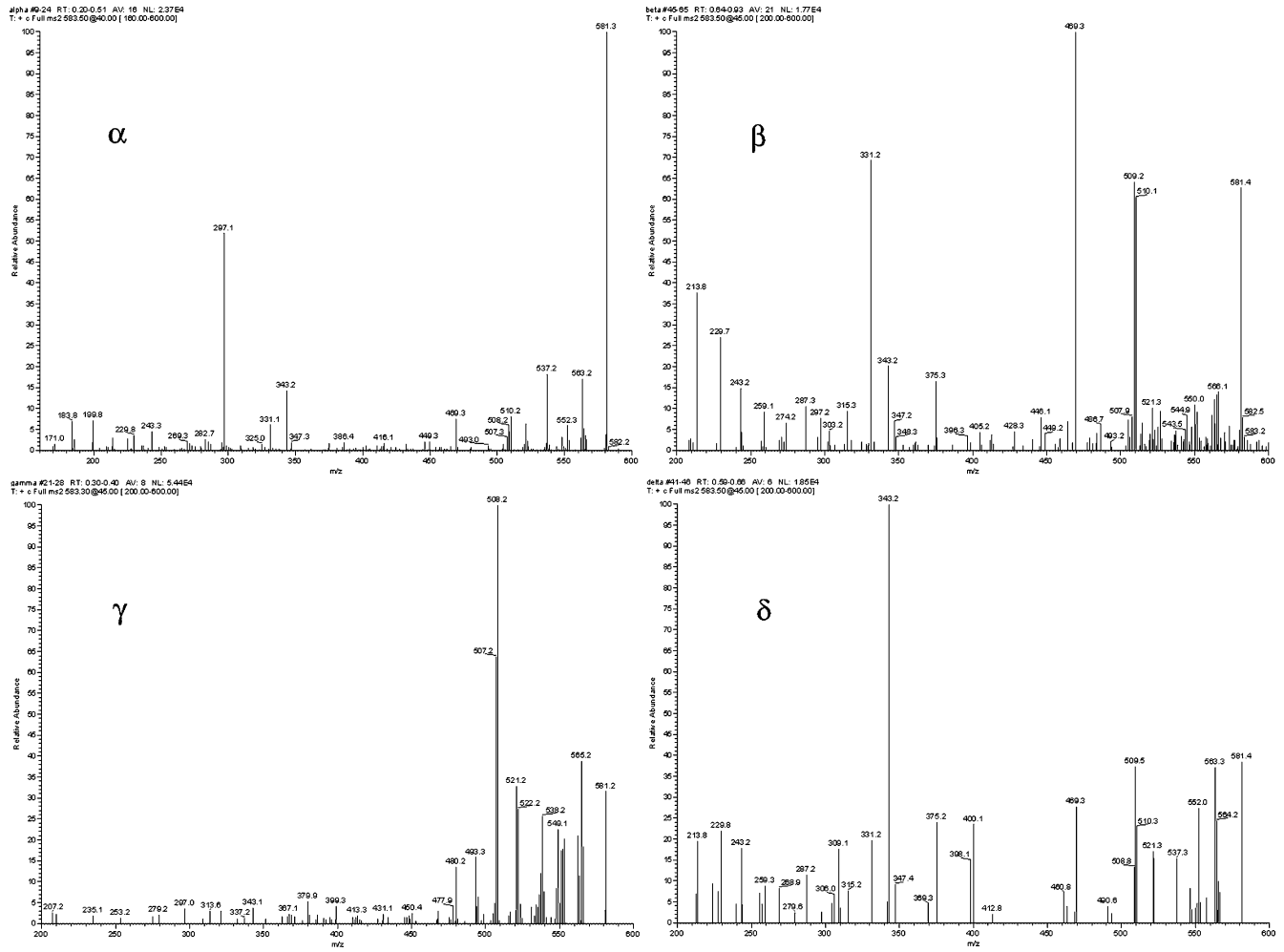

Figure 5. MS/MS analysis of the four biliverdin IX regioisomers.

The commercially available biliverdin that was used for product identification with LC-MS was biliverdin IXα. However, the reaction could occur at any or all meso positions of the heme, leading to biliverdin IXα, *β*, *γ*, or *δ*. α-Meso oxidation specificity is a key feature in the heme oxygenase-dependent conversion of heme to biliverdin IXα,[17] but the other three isomers are found as heme metabolites in lower animals,[18] in body fluids of mammals,[19] in the bile of mutant rats that cannot esterify bilirubin IXα,[20] with mutated heme oxygenase,[21] and when meso-substituted hemes are used as heme oxygenase substrates.[14,22] The MS/MS spectrum of the unknown biliverdins from heme degradation had a base peak of 297 (Figure 3A), which appears to represent half of the biliverdin product (Figure 4). To keep the mass values of the two "halves" of biliverdin odd, the four nitrogen atoms have to be divided into two and two. A comparison of the structures of the four possible

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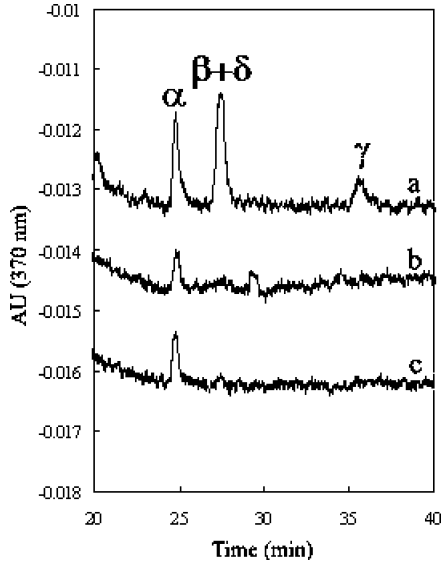

Figure 6. HPLC analysis (370 nm) of the biliverdins from iNOS inactivation. (a) Four synthetic biliverdin IX regioisomers; (b) biliverdin from iNOS inactivation with 1400W (the tiny peak at 29.3 min is heme); (c) biliverdin from iNOS inactivation with L-NIO.

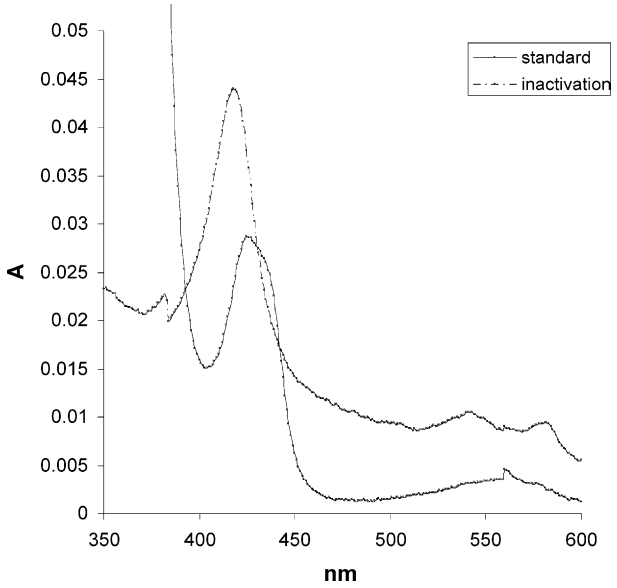

Figure 7. Formation of CO during iNOS inactivation by L-NIO or 1400W using deoxymyoglobin as the trapping reagent.

biliverdin IX isomers indicates that to accommodate this base peak, the β- and δ-isomers must be excluded.

The identification of the structure of the biliverdin formed in heme oxygenase-catalyzed reactions normally can be done in a relatively straightforward manner because the quantity of biliverdin produced from heme as a substrate is sufficient for identification by two-dimensional NMR[21] or by conversion to the corresponding dimethyl esters, which were identified by comparison of HPLC retention times with the four synthetic biliverdin IX dimethyl esters.[14,23] The biliverdins produced in our studies is the degradation products of the heme cofactor in iNOS, and the small amount produced (about 0.1−2.0 μM) for analysis leads to inconclusive results by NMR methods. Consequently, our identification relied on mass spectral approaches.

The four biliverdin IX isomers were synthesized from a coupled-oxidation reaction[24] and were used as reference standards to identify the biliverdin produced by iNOS inactivation. It is apparent from the mass spectra of the four biliverdin isomers under the same analysis conditions (Figure 5) that the only isomer whose mass spectrum matches the biliverdin produced by 1400W and L-NIO inactivation of iNOS is biliverdin IXα (although the γ-isomer also has the same fragmentation pattern theoretically, it might require a different collision energy). Furthermore, conditions were developed to separate the four biliverdin isomers into three peaks by HPLC (Figure 6), and confirmation was obtained that the biliverdin produced in the enzyme inactivation is biliverdin IXα. The identification of the regioisomer of the biliverdin produced from iNOS inactivation by 1400W and L-NIO is important to the elucidation of the proposed heme degradation mechanism and will be very helpful for the elucidation of the interaction between the inactivators and the active site of the enzyme.

The α-regiospecificity of the heme conversion to biliverdin by iNOS incubation with amidines is the same as that observed in the heme oxygenase-catalyzed conversion of substrate heme and suggests that a heme oxygenase-like mechanism for iNOS inactivation by 1400W and L-NIO may be relevant. Because both heme and α-*meso*-methylheme are substrates for heme oxygenase and both produce biliverdin IXα,[14] we previously proposed two possible inactivation mechanisms for L-NIO inactivation of iNOS.[7] In one of the inactivation mechanisms, the amidine group of the inactivator is first oxidized by NOS, which then covalently binds to the α-meso-position of the heme to form an α-*meso*-alkylated heme (Scheme 2). This change in the NOS oxygenase domain converts it into an "NOS heme-oxygenase domain" which is capable of catalyzing the degradation of this α-*meso*-alkylated heme to biliverdin similarly to the reaction of α-*meso*-methylheme with heme oxygenase. This reaction would convert the α-*meso*-1400W-heme or α-*meso*-L-NIO-heme into verdoheme (**14**) and carboxylated 1400W or carboxylated L-NIO. These β-imino carboxylic acids should undergo rapid and spontaneous decarboxylation to regenerate 1400W or L-NIO, which would account for why we[7] and others[6] have found that these inactivators do not appear to be modified during inactivation.

In the second of the proposed inactivation mechanisms, the binding of the amidines to NOS results in uncoupling of the hydrogen peroxide produced, which may somehow lead to the formation of α-*meso*-hydroxyheme, decomposing to biliverdin and CO, as in the case of heme oxygenase.

Whereas the observed deuterium isotope effects with CD_3-1400W and CD_3-NIO are easily rationalized by the first mechanism (Scheme 2), which involves initial methyl C−H bond cleavage, it would be difficult to rationalize the deuterium isotope effect by a mechanism that involved uncoupling of hydrogen peroxide. Furthermore, previous studies argue against the hydroperoxide overproduction mechanism: (1) Although hydrogen peroxide degrades free heme and cytochrome P450 bound heme, the products of peroxidative heme cleavage consisted of various propentdyopents, maleimides, formic acid (the *meso*-carbons), and hematinic acid; biliverdin, however,

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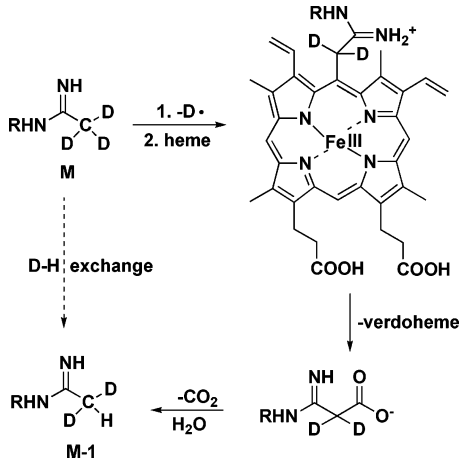
Scheme 5



was not a product or intermediate in this process;[25] (2) tetrahydrobiopterin, one of the cofactors included in the inactivation mixture, was an inhibitor of the superoxide formation process by a mechanism that does not cause inhibition of NADPH consumption;[26,27] (3) inclusion of catalase does not prevent significant heme loss during iNOS incubation with L-NIO, which suggests that heme loss is not related to high concentrations of hydroperoxide.[7]

The alkylated heme mechanism (Scheme 2) can be tested. As shown in Scheme 5, oxidation of CD_3-1400W or CD_3-NIO removes one of the three methyl deuteriums prior to alkylation of the heme. Following oxidation of the inactivator-bound heme and formation of the carboxylated inactivator, decarboxylation results in the formation of the starting inactivator (1400W or NIO), but with only two deuteriums in the methyl group. Mass spectrometry can determine if one deuterium was lost. The amount of inactivator used in the experiment was only in slight excess (heme:inactivater = 1:5) to keep inactivation fast and to amplify the possible H−D exchange for easy mass spectral detection. Derivatization of the amidines after inactivation with *o*-phthalaldehyde/dithiothreitol reagent made their detection straightforward. LC-MS of the derivatized inactivators clearly showed that H−D exchange does not accompany iNOS inactivation; no CHD_2-1400W or CHD_2-NIO was detected. The alkylated heme mechanism (Scheme 2) is not consistent with this result and is disproved.

This finding is consistent with the crystal structure of eNOS-L-NIO,[28] which shows that the amidine methyl carbon of L-NIO is 3.95 Å from the α-*meso*-carbon of heme. This is too far for formation of the alkylated α-*meso*-heme required for the mechanism in Scheme 2. For bond formation to occur, there has to be a movement of the inactivator toward the heme α-meso position. This movement would break the hydrogen bonding between the amidine nitrogen atoms and the glutamic acid residue (E363) of eNOS, which is a high-energy barrier to cross. Furthermore, the hydrophobic area around the heme α-*meso*-carbon would not be sufficiently spacious to accommodate the inactivator-heme adduct.

Because the alkylated heme mechanism (Scheme 2) is not supported by our results and the excess hydrogen peroxide mechanism is not consistent with previous results that show that hydrogen peroxide does not convert heme to biliverdin and also does not rationalize the deuterium isotope effect on the methyl C−H bond during inactivation, a third mechanistic possibility is required. If the inactivation mechanism is related to the mechanism of heme oxygenase-catalyzed conversion of heme to biliverdin, then carbon monoxide should be the other product.[13] To test this, the inactivation of iNOS by either 1400W or L-NIO was carried out in a capped vial in the presence of a limiting amount of freshly prepared ferrous deoxymyoglobin as the CO trapping reagent in a separate tube inside the vial. As shown in Figure 7, CO is produced by iNOS inactivation with both L-NIO and 1400W, which strongly supports a mechanism related to the heme oxygenase mechanism. This also confirms the conclusion that an alkylated heme is *not* involved in the inactivation of iNOS by amidines, because heme oxygenase converts α-methylheme to biliverdin, but without production of CO.[14]

It appears, then, that the inactivation of iNOS by amidines is related to the mechanism of heme oxygenase oxygenation of heme to biliverdin. The amidines have a strong structural relationship to L-arginine, and crystal structures of L-NIO and 1400W bound to all isozymes of NOS[28,29] show that they all bind similarly. This suggests that possibly the abnormal behavior of amidines is a result of both the absence of the guanidino group of L-arginine as well as the presence of the amidino group of L-NIO and 1400W.

Speculation about the role of the amidino group in NOS inactivation begins with a comparison of various heme-dependent enzyme mechanisms. Consider the mechanisms of cytochrome P450, NOS, and heme oxygenase. All three of these enzymes catalyze oxygenation of substrates, and all three are believed to proceed via a heme peroxide intermediate (Figure 8). In cytochrome P450, an amino acid residue in the active site protonates the distal peroxide oxygen atom, which leads to the loss of water and the formation of the high-energy iron-oxo species (known as compound I).[30] This is the same mechanism proposed for the NOS-catalyzed oxygenation of L-arginine to N^{ω}-hydroxy-L-arginine,[31] except that the proton donor was proposed to be the guanidino amino group of the substrate L-arginine.[28] This conclusion is based on the crystal structure of the NO complex of eNOS with L-arginine bound,[28] where it is apparent that the guanidino amino group is hydrogen bonded to the distal oxygen atom of NO, and no other active site proton donors are available. In the heme oxygenase, the proton donor is an active site residue, but it is close to the proximal peroxide oxygen rather than to the distal oxygen.[32] This allows a reaction to occur between the heme porphyrin ring and the distal peroxide oxygen, leading to α-*meso*-hydroxyheme and then on to biliverdin via verdoheme.

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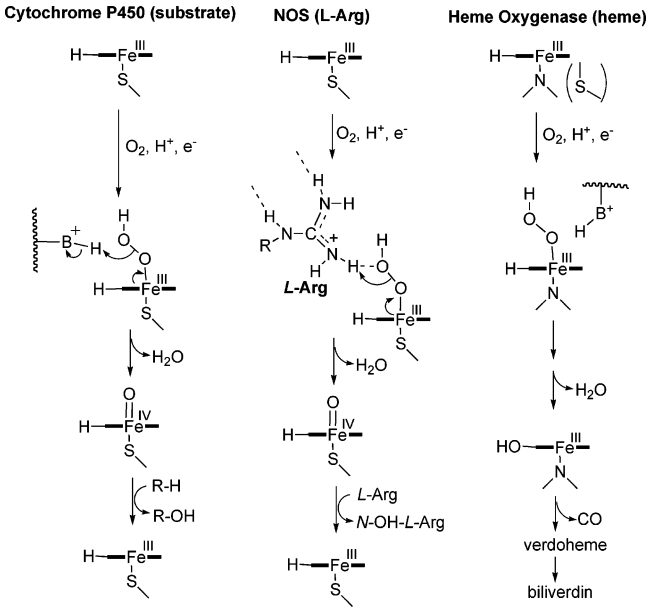

Figure 8. Comparison of the mechanisms for cytochrome P450, nitric oxide synthase, and heme oxygenase.

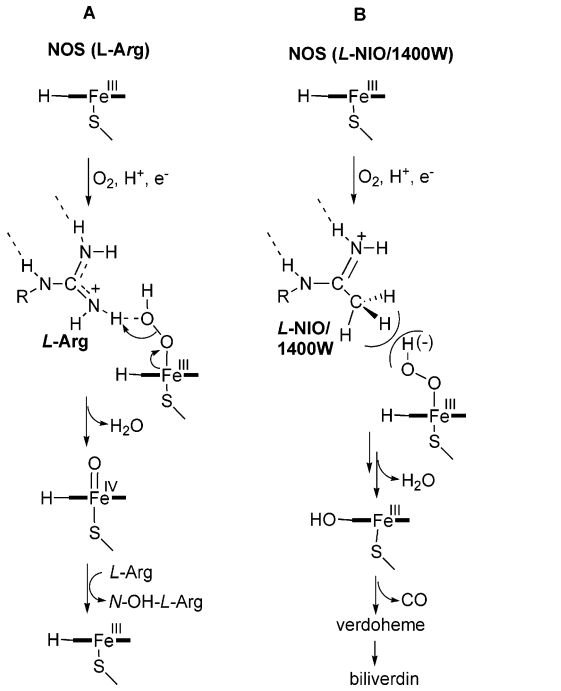

Figure 9. Comparison of the reaction of NOS with L-arginine bound and the proposed mechanism when L-NIO or 1400W is bound.

The active site region of all three isozymes of NOS around the arginine-binding site is hydrophobic.[28,29] The crystal structure with L-NIO bound[28] shows that the amidino methyl group faces inward toward the heme peroxide, making the active site even more hydrophobic. A water molecule that is observed between the guanidino group of bound L-arginine and the proline residue in the active site of eNOS is not present in the complex between eNOS and L-NIO, presumably because of the increased hydrophobicity with the amidine bound.[28] This hydrophobicity of the active site coupled with the fact that the amidino methyl group of the inactivators replaces the guanidino amino group of L-arginine that provides the proton for activation of the heme peroxide (or peroxy anion) rationalizes why the amidines convert NOS catalysis to a heme oxygenase reaction (Figure 9).

Because there is no longer a proton source for conversion of the heme peroxide (or peroxy anion) into the iron-oxo species (the amino group of the guanidine is replaced by the methyl group of the amidine) and the amidino methyl group is not sufficiently activated for hydroxylation, the enzyme resorts to the alternative heme oxygenase reaction. This leads to the formation of α-*meso*-hydroxyheme, which then is converted to verdoheme and biliverdin by further oxidation.

This proposed mechanism accounts for all of the experimental observations except for the deuterium isotope effect on inactivation by the trideuteriomethylated amidines. If the mechanism of inactivation of NOS by 1400W and L-NIO does not require cleavage of the methyl C−H bond, then why is there a deuterium isotope effect of about 1.3 on inactivation? Two effects may be responsible. A CD_3 group is slightly smaller than a CH_3 group,[33] and deuterium is less hydrophobic than hydrogen.[34] Because both closeness of packing and hydrophobicity appear to be important to the proposed inactivation mechanism, both of these properties could easily be responsible for the small kinetic isotope effect on inactivation.

The mechanism proposed for amidine inactivation of NOS is quite novel. Irreversible inactivators typically cause inactivation by becoming covalently attached to the enzyme or, at least, by undergoing a catalytic reaction to produce a molecule that causes irreversible inactivation. We have not been able to identify another example of a compound that causes irreversible inactivation of a wild-type enzyme that does not itself become modified, as is the case with the amidine inactivators of NOS.

In conclusion L-NIO, 1400W, and possibly all amidine-containing inactivators of NOS appear to inactivate that enzyme by preventing protonation of the heme peroxide intermediate, thereby preventing the formation of the heme iron-oxo species and normal substrate oxygenation. The alternative pathway, one that is related to the reaction catalyzed by heme oxygenase, results in self-mutilation and irreversible inactivation of NOS by conversion of its heme cofactor into biliverdin.

Experimental Section

General Methods and Reagents. Optical spectra and enzyme assays were recorded on a Perkin-Elmer Lambda 10 UV/vis spectrophotometer. HPLC analyses were carried out on a Beckman 125 Solvent Module delivery system equipped with a Beckman 166 detector. All NMR spectra were recorded on a Mercury 400 spectrometer. Chemical shifts (δ) are reported downfield from tetramethylsilane (Me_4Si) in parts per million (ppm). Mass spectra were recorded on G70-250SE (ESI), Finnigan MAT900XL (EI), or Micromass Quattro II (APCI) spectrometers.

Flash chromatography was performed with Merck silica gel (230−400 mesh). TLC plates (silica gel 60-F254) were purchased from VWR Scientific. L-Arginine, NADPH, HEPES, DTT, human ferrous hemoglobin, OPA reagent, and myoglobin (from horse skeletal muscle) were purchased from Sigma Chemical Co. Hemin and biliverdin IXα were purchased from ICN Pharmaceuticals, Inc. Tetrahydrobiopterin (BH_4) was purchased from Alexis Biochemicals. 1-(*N*-Boc-aminomethyl)-3-(aminomethyl)benzene was purchased from Aldrich Chemical Co. CD_3-CN was purchased from Acros Organic Co. L-NIO was purchased from Research Biochemicals International. 1400W was either purchased from

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Alexis Biochemicals or synthesized. Acids, bases, and conventional organic solvents were purchased from Fisher.

Expression and Purification of Recombinant iNOS. A recombinant murine iNOS/calmodulin overexpression system in *Escherichia coli* was obtained, and the enzyme was purified.[35] The enzyme was quantified according to the methods of Stuehr and Ikeda-Saito[36] using $\epsilon_{397} = 71\ mM^{-1}\ cm^{-1}$ and a MW of 150 kDa.

Hemoglobin Assay for iNOS Activity. The production of nitric oxide by iNOS was measured by the rapid oxidation of oxyHb to metHb by nitric oxide.[37] The assay mixture contained iNOS stock or aliquots from incubation, L-arginine (0.1 mM), NADPH (0.1 mM), tetrahydrobiopterin (10 μM), dithiothreitol (100 μM), and oxyhemoglobin (7.5 μM), diluted to a total volume of 600 μL with Hepes buffer (100 mM, pH 7.4). The relative rate of nitric oxide synthesis was determined by monitoring the NO-mediated conversion of oxyhemoglobin to methemoglobin at 401 nm on a Perkin-Elmer Lambda 10 UV/vis spectrophotometer. All assays were performed at 30 °C.

HPLC of Products formed from 1400W Inactivation of iNOS. Chromatograms of iNOS inactivated by 1400W (107 μM) were obtained on a C18 reversed-phase column (Vydac, 218TP54, 5 μm, 4.6 × 250 mm) at 401 nm with 60% H_2O (0.1% TFA) and 40% CH_3CN (0.1% TFA). The flow rate was 1.0 mL/min. Control was made by either omitting NADPH or 1400W. Standards were made with commercial biliverdin IXα and hemin.

LC-Electrospray Mass Spectra of Inactivation Mixtures. iNOS incubation with 1400W (107 μM) and other cofactors was kept at 37 °C for 2 h. A control was made by omitting the 1400W. A standard sample was made with commercial biliverdin IXα and hemin. Aliquots of 20 μL of these three samples were injected directly onto a YMC ODS-AQ C_{18} column (2.0 × 250 mm). A Waters 2690 solvent delivery system was used to elute the following gradient with a rate of 200 μL/min: 35−55% B over 10 min followed by 55−75% over 25 min with solvent A consisting of water and solvent B of CH_3CN. All solvents contained 0.1% formic acid. After 8 min, the column outlet was attached to a Q-TOF-2 quadrupole/time-of-flight mass spectrometer without splitting for monitoring column output using positive mode ionization. The scan range was 300−750, and the collision energy for the MS/MS experiment was 35 eV.

Synthesis of the Dimethyl Esters of Biliverdin IX Isomers. The dimethyl esters of biliverdin IX isomers were obtained by oxidative cleavage of hemin with O_2-ascorbic acid followed by hydrolysis with methanolic KOH and esterification with BF_3-MeOH, as described by Bonnett and McDonagh.[24]

Separation of the Dimethyl Esters of Biliverdin IX Isomers. The biliverdin dimethyl ester isomers were isolated by preparative TLC according to the method described by Heirwegh et al.[38] with slight modification. The mixture of the four esters was eluted five times with chloroform/acetone (39:1, v/v) on a 20 × 20 cm preparative TLC plate, and the four esters were distinctly separated into four bands with R_f values of 0.62 (δ), 0.68 (γ), 0.72 (α), and 0.75 (β). The identity of the compounds in the middle two bands was further confirmed by HPLC (see Supporting Information): the retention time of the R_f 0.72 band is the same as biliverdin IXα dimethyl ester, which was prepared from commercial biliverdin IXα and BF_3-MeOH;[24] the R_f 0.68 band has the longest retention time, which is consistent with all of the previous reversed-phase HPLC analysis results.[23,39] MS data showed M + H^+ peaks of all four bands to be 611.5 (611.7 calculated).

Preparation of the Free Acids of Biliverdin IX. The four bands from preparative TLC were scraped off, and the esters were dissolved in ethyl acetate/hexane (2:1, v/v). After concentration by rotary evaporation, the residues (5−20 mg) were dissolved in 10 mL of MeOH, flushed with argon, and mixed with 5 mL of 1 M NaOH containing 1 mM Na_2EDTA. The mixture was kept at 37 °C for 1 h for the α-, β-, and δ-isomers and overnight for the γ-isomer. After the reaction was complete, 5 mL of glycine/HCl (pH 1.8) was added to each, followed by 5 mL of 1 M HCl. The acidified mixture was then extracted with an equal volume of CH_2Cl_2 and was dried over anhydrous Na_2SO_4 before rotary evaporation. In the ^1H NMR spectra (CD_3OD) of the four biliverdin IX isomers, the peaks for the methyl ester hydrogens (s, 3.66−3.72) disappeared after saponification. MS data showed the M + H^+ peak of all four hydrolyzed products to be 583.3−583.5 (583.7 calculated).

MS/MS Analysis of the Four Biliverdin IX Isomers. Deprotonated molecules of *m/z* 581 were detected for the biliverdin IX isomers using negative ion mode mass spectrometry (Figure 5). Electrospray was used for the analysis of the α-, β-, and δ-isomers on a VG70-250SE high-resolution mass spectrometer and APCI for the γ-isomer on a Micromass Quattro II triple quadrupole mass spectrometer. Commercial biliverdin IXα was used to tune the instruments, and then MS-MS spectra were obtained for all four isomers. The collision energy for collision-induced dissociation during MS/MS was 8 kV for the VG70-250SE instrument and 35 eV for the Quattro II.

Biliverdin Identification by HPLC Analysis. HPLC analysis was performed using the same conditions for the analysis of the iNOS inactivation mixture except for a different elution program: 100% A for 2 min followed by 0−100% B over 10 min with solvent A consisting of H_2O (0.1% TFA) and solvent B of 25% KH_2PO_4 solution (20 mM, pH 4.0) and 75% MeOH. The elution was monitored at 370 nm with a flow rate of 0.5 mL/min.

Calibration Curves of Biliverdin and Heme and the Calculation of the Ratio of Biliverdin/Heme Loss. Commercial biliverdin IXα and hemin were dissolved in methanol to make a concentration of 1.0 mM, which was then diluted with H_2O. A mixture of biliverdin and heme in H_2O (1.0, 2.0, 3.0, 4.0, 5.0 μM, each) was analyzed using the same conditions as the HPLC analysis of the iNOS inactivation mixture. The area of each peak was calculated with the Beckman Gold Chromatography System (Version 1.6). Calibration curves of biliverdin and heme were determined by plotting HPLC peak area versus concentration (see Supporting Information). iNOS inactivation by L-NIO was determined by replacing 1400W with L-NIO. Heme loss was calculated by comparing the heme concentration in the control and inactivation mixtures.

***N*-(3-(Aminomethyl)benzyl)acetamidine (1400W, 4).** Acetimidic acid ethyl ester hydrochloride (247 mg, 2.00 mmol), prepared according to the method of Liu et al.,[40] was dissolved in 10 mL of ethanol, and then 567 mg (2.40 mmol) of 1-(*N*-Boc-aminomethyl)-3-(aminomethyl)-benzene was added all at once. The reaction mixture was stirred at 0 °C for 3 h and then partitioned between 20 mL of diethyl ether and 20 mL of H_2O. The aqueous layer was washed with 10 mL of diethyl ether twice before concentration by high-vacuum rotary evaporation to a dry white solid of *N*-(3-(*N*-Boc-aminomethyl)benzyl)-acetamidine hydrochloride (577 mg, 1.84 mmol, 92%): ^1H NMR (D_2O) d 1.41 (s, 9H), 2.28 (s, 3H), 4.24 (s, 2H), 4.48 (s, 2H), 7.18−7.42 (m, 4H).

The above compound (160 mg, 0.51 mmol) was stirred in a mixture of 30 mL of 1,4-dioxane and 4 mL of 4 N HCl at ambient temperature for 3 h. The reaction mixture was dried by high-vacuum rotary evaporation to yield *N*-(3-(aminomethyl)benzyl)acetamidine dihydrochloride (125 mg, 0.50 mmol, 98%) which was further purified by three consecutive recrystallizations with ethanol and diethyl ether to give the product as a white granular crystalline solid: ^1H NMR (D_2O) d 2.27 (s, 3H), 4.18 (s, 2H), 4.52 (s, 2H), 7.30−7.52 (m, 4H); HRMS (EI): calcd for $C_{10}H_{15}N_3$, $M^+_{calcd} = 177.1260$, $M^+_{obs} = 177.1261$.

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***N*-(3-(Aminomethyl)benzyl)-2-[$^2H_3$]-acetamidine (d_3-1400W, 11).** d_3-1400W (126 mg, 0.50 mmol, 90% overall) was synthesized by the same route used to make 1400W except that CD_3CN was substituted for CH_3CN to make 2-[$^2H_3$]-acetimidic acid ethyl ester hydrochloride: ^1H NMR (D_2O) d 4.18 (s, 2H), 4.52 (s, 2H), 7.30−7.52 (m, 4H); HRMS (EI): calcd for $C_{10}H_{12}{}^2H_3N_3$, $M^+_{calcd} = 180.1448$, $M^+_{obs} = 180.1449$.

Irreversible Inhibition Kinetics. iNOS (0.1−0.3 μM) was incubated at 37 °C in Hepes buffer (100 mM, pH 7.4), containing catalase (500−1000 units), NADPH (9 mM), tetrahydrobiopterin (1 mM), dithiothreitol (10 mM), glycerol (10%, v/v), and inactivator in a total volume of 70−280 μL. The reactions were initiated by the addition of iNOS, and 10 μL aliquots were removed to assay for enzyme activity at various times. Controls were made by omitting the inactivators or NADPH.

LC-MS of OPA-DTT-derivatized d_3-1400W after iNOS Inactivation. OPA-DTT reagent was prepared by mixing *o*-phthalaldehyde (OPA, 1 mg/mL, "incomplete") reagent with DL-dithiothreitol (DTT) to a concentration of 100 mM. Inactivation mixtures were prepared as described before in a volume of 210 μL with 5 μM or 10 μM d_3-1400W. Controls were prepared by omitting the enzyme. After the enzyme fully lost its activity, the mixture (200 μL) was diluted with Hepes buffer (100 mM, pH 7.4) to 1.0 mL, which was then ultrafiltered with a stirring cell (NMWL: 10 000). The filtrate was collected, and 50 μL of the solution was derivatized with 10 μL of OPA-DTT reagent for 5 min. HPLC analysis confirmed that d_3-1400W was fully recovered by comparing the adduct peak with that of the control. LC-MS (APCI detector) studies were performed on the OPA-DTT-derivatized inactivation mixture and control in parallel.

Carbon Monoxide Detection. Into a 10-mL vial was added 82.8 μL of glycerol, 192.0 μL of NADPH (39.0 mM), 94.2 μL of tetrahydrobiopterin (8.0 mM), 403.8 μL of iNOS (2.0 μM), and a stirring bar. A small thin tube (made by sealing the tip of a piece of Pasteur pipet) containing 55.0 μL of freshly prepared ferrous deoxymyoglobin (2.0 μM), prepared by adding sodium dithionite (5 mg) to an 80.0 mL solution of horse skeletal muscle myoglobin (2.7 mg, 16.0 nmol) in 100 mM Hepes buffer (pH 7.4), was carefully put into the 10 mL vial, which was sealed with a rubber septum immediately. The 1400W solution (67.2 μL, 500 μM) was injected into the enzyme solution via a syringe, and the mixture was incubated at 37 °C with mild stirring for 30 min. After removal of the septum, 50.0 μL of the ferrous deoxymyoglobin solution was diluted to 500.0 μL with Hepes buffer (100 mM, pH 7.4), and the spectrum was recorded against Hepes buffer. The same experiment was repeated on iNOS inactivated with L-NIO (7.5 mM). A control was made by replacing the 1400W or L-NIO with Hepes buffer.

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Supporting Information Available: Six figures of data and a scheme. This material is available free of charge via the Internet at http://pubs.acs.org.

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