Structural Requirements for Human Inducible Nitric Oxide Synthase Substrates and Substrate Analogue Inhibitors

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ABSTRACT: Inducible nitric oxide synthase (iNOS; EC 1.14.13.39) catalyzes the NADPH-dependent oxidation of one of the free guanidino nitrogens of L-Arg to form nitric oxide and L-citrulline. Analogues of L-Arg and the inhibitor, L-N^6-(1-iminoethyl)lysine, were used to define structural elements required for the binding and catalysis of compounds. L-Arg analogues with sequentially shorter methylene spacing between the guanidino group and the amino acid portion of the molecule were not iNOS substrates but were reversible inhibitors. L-Arg analogues such as agmatine with a hydroxyl substitution at the 2-amino position were substrates. Desaminoarginine was not a substrate but a reversible inhibitor. Desaminoarginine, agmatine, and argininic acid bound to the enzyme to give type I difference spectra similar to that of L-Arg. The amidino compounds L-N^6-(1-iminoethyl)lysine, L-N^5-(1-iminoethyl)ornithine, and N^5-(1-iminoethyl)cadaverdine, but not N^6-(1-iminoethyl)-6-aminocaproic acid, were NADPH-dependent, irreversible inactivators of iNOS. For both the L-Arg and L-N^6-(1-iminoethyl)lysine analogues, the 2-amino group appeared to play an important role in catalytic events leading to either substrate turnover or mechanism-based inactivation. Inactivation of iNOS by L-N^6-(1-iminoethyl)lysine was NADPH- and dioxygen-dependent, but low incorporation of radiolabel with DL-[4,5-^{3}H]-N^6-(1-iminoethyl)lysine indicates that the mechanism of enzyme inactivation is not covalent modification of the protein.

Inducible nitric oxide synthase (iNOS;[1] EC 1.14.13.39) is a heme-containing flavoprotein which catalyzes the oxidization of L-Arg utilizing NADPH and dioxygen to form nitric oxide (NO) and L-citrulline. Both L-homoarginine (*1*, *2*) and L-N^G-hydroxyarginine are also iNOS substrates, and the latter is the proposed intermediate in the overall oxidation of L-Arg to NO and L-citrulline (*3*−*5*). The enzyme displays enantioselectivity for the L-form of amino acid substrates (*1*, *2*). *N*-Guanidino derivatives of L-Arg can act as either competitive, reversible inhibitors of iNOS, such as L-N^G-nitroarginine (*6*), or irreversible inhibitors, such as L-N^G-methylarginine (*7*, *8*). Amidino amino acids are also inhibitors of iNOS, such as L-N^6-(1-iminoethyl)lysine (L-NIL) (*9*) and L-N^5-(1-iminoethyl)ornithine (L-NIO) (*10*). For these iNOS substrate analogue inhibitors, the L-enantiomer of the amino acid is also essential for their inhibitory activity (*9*). This selectivity is intriguing when one considers that small non-amino-acid guanidines (*11*) and amidines (*12*, *13*) are very potent inhibitors of iNOS. We therefore became interested in identifying structural elements of compounds that are required for binding to iNOS and whether these requirements are also essential for catalysis. Specifically, we have examined a series of L-Arg and L-NIL analogues which either differ in the carbon chain length between the guanidino portion of the molecule and the amino acid or lack the 2-amino or 2-carboxy group of the amino acid. We found that the methylene spacing and the functional groups at the 2-position are essential structural elements for both substrate turnover and NADPH-dependent inactivation of enzyme activity.

EXPERIMENTAL PROCEDURES

Reagents and Proteins. *N*-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES), L-arginine hydrochloride, NAD, NADH, NADP, NADPH, FAD, FMN, BSA, agmatine sulfate, L-2-amino-3-guanidinopropionic acid hydrochloride, L-argininic acid, 2-chloro-5-guanidino-*n*-valeric acid hydrochloride, L-citrulline, L-homoarginine hydrochloride, and bovine calmodulin (CaM) were purchased from Sigma. Desaminoarginine hydrochloride was purchased from Peninsula Labs (Belmont, CA). 6(*R*)-5,6,7,8-Tetrahydrobiopterin (BH_4) was purchased from Dr. B. Schircks (Jona, Switzerland). Carbon monoxide gas, imidazole, sodium nitrite, and ammonium iron(II) sulfate hexahydrate were purchased from Aldrich. L-N^6-(1-Iminoethyl)lysine hydrochloride and L-N^5-(1-iminoethyl)ornithine were purchased from Alexis Corp. (San Diego, CA). L-[2,3,4,5-^{3}H]Arginine hydrochloride (57 Ci/mmol) and L-[2,3,4,5-^{3}H]citrulline (44 Ci/mmol) were purchased from Amersham (Arlington Heights, IL).

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[1] Abbreviations: NO, nitric oxide; iNOS, inducible nitric oxide synthase; rh-iNOS, recombinant human iNOS; m-iNOS, murine macrophage iNOS; L-NIL, L-N^6-(1-iminoethyl)lysine; L-NIO, L-N^5-(1-iminoethyl)ornithine; NMA, L-N^G-methylarginine; TES, *N*-[tris-(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; CaM, calmodulin; BH_4, 6(*R*)-5,6,7,8-tetrahydrobiopterin; DTT, DL-dithiothreitol; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.

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L-N^2-Methylarginine was synthesized according to a literature procedure (*14*). DL-[4,5-^{3}H]-N^6-(1-Iminoethyl)-lysine was prepared by reacting DL-dehydrolysine (Bachem) with ethyl acetamidate hydrochloride at pH 9 as described for the preparation of L-NIL (*15*). The resulting DL-dehydro-NIL was reacted with tritium in dimethylformamide with a palladium/carbon catalyst by the radiochemical synthesis group at Merck (Rahway, NJ). The radiochemical purity was judged to be 99.2% by HLPC analysis and was used as a tracer with L-N^6-(1-iminoethyl)lysine hydrochloride. L-2-Amino-4-guanidinobutanoic acid was synthesized from homoserine. Displacement of the hydroxyl group of Boc-L-homoserine *tert*-butyl ester by di-Boc-guanidine under Mitsunobu conditions as described by Kozikowski (*16*) and removal of protecting groups by HCl in ethyl acetate furnished L-2-amino-4-guanidinobutanoic acid as a bis hydrochloride salt. To prepare N^6-(1-iminoethyl)-6-amino-caproic acid (desamino-NIL), *tert*-butyl 6-(thioacetylamino)-hexanoate was reacted with $HgCl_2$, NH_3 to form the corresponding acetamidine and the *tert*-butyl ester was removed by HCl in dioxane. In an analogous protocol, 6-(Boc-amino)-1-(thioacetylamino)hexane was converted to its acetamidine by $HgCl_2$, NH_3 and the Boc protecting group was removed by HCl to obtain N^5-(1-iminoethyl)cadaverdine (descarboxy-NIL).

Recombinant human inducible NOS (rh-iNOS) was purified by immunoaffinity chromatography as described previously (*17*).

Enzyme Assays. Enzyme L-citrulline production was assayed at room temperature (22 °C) in 50 μL of assay buffer containing 0.1 M TES (pH 7.5), 1 μM L-Arg, 4 μCi/mL L-[2,3,4,5-^{3}H]Arg, 5 μM FAD, 5 μM FMN, 0.5 mM NADPH, 0.2 mg/mL BSA, 10 μg/mL CaM, 10 μM BH_4, 0.25 mM DTT, 2 mM $CaCl_2$, and 2−10 μg of enzyme. Quantitation was performed by HPLC separation of L-[2,3,4,5-^{3}H]citrulline and L-[2,3,4,5-^{3}H]Arg as previously described (*18*). Nitrite in solution was detected with the Griess reagent (*19*). Potential substrates were tested at 1 mM with 5 μg of purified iNOS in 50 μL of 0.1 M TES (pH 7.5), 5 μM FAD, 5 μM FMN, 10 μM BH_4, 0.25 mM DTT, 2 mM NADPH, 0.2 mg/mL BSA, 10 μg/mL CaM, and 2 mM $CaCl_2$. Reactions were incubated at 25 °C for 4 h in triplicate and quenched with equal volumes of the Griess reagent. Absorbance at 540 nm was quantitated to a standard curve with sodium nitrite. Note that all enzyme assays were under aerobic conditions containing NADPH unless specifically mentioned. The test of dioxygen dependence for enzyme inactivation by L-NIL was performed anaerobically by sparging all solutions under dry argon with the addition of 0.2 mM ammonium Fe(II) sulfate and 2 mM DTT to scavenge residually dissolved oxygen (*20*). This amount of ammonium Fe(II) sulfate inhibited iNOS activity by 25%.

Radiolabeling Studies. Radiolabeling studies were performed in duplicate under the following conditions: 0.5 mL containing 50 mM TES (pH 7.5), 0.2 M NaCl, 0.5 mM NADPH, 0.25 mM DTT, 10 μM BH_4, 5 μM FAD, 5 μM FMN, 128 μg iNOS, 10 μM L-NIL, and 20 μCi of DL-[4,5-^{3}H]NIL at 25 °C for 90 min. Samples were dialyzed over 5 h at 4 °C against three changes of 0.5 L of buffer containing 20 mM TES (pH 7.5), 50 mM NaCl, 0.5 mM DTT, 0.1 mM BH_4, 2 μM FAD, and 2 μM FMN, at 4 °C. Slide-A-Lyzer cassettes from Pierce (Rockford, IL) were used for the dialysis of protein samples. Samples (0.35 mL) were added to 3 mL of scintillation cocktail with an average of 4833 ± 909 cpm for the samples compared to the final dialysis buffer with 45 cpm.

Kinetic Analysis. Linearity of iNOS L-citrulline production was confirmed by initial velocity measurements for up to 90 min. Time-dependent inhibition was evaluated by fitting nonlinear progress curves to the first-order expression: $y = a_0(1 - e^{-kt})$, where y is L-citrulline concentration at each time point, a_0 is the total amount of product formed, k is the observed first-order rate constant, k_{obs}, and t is assay time. Kinetic parameters for inactivation were determined from replots of $1/k_{app}$ (or $t_{1/2}$) as a function of reciprocal inhibitor concentration (*21, 22*), where k_{app} is the slopes of the curves obtained from the inactivation plots fitted to the integrated first-order expression: $y = v_s t + [(v_0 - v_s)(1 - e^{-k_{app}t})/k_{app}] + a_0$. NADPH-dependent enzyme inactivation was evaluated by preincubation of enzyme with inhibitor at its IC_{50} concentration in the presence or absence of 0.5 mM NADPH. Residual iNOS activity was then determined after dilution (1:10) into assay buffer containing 10 μM L-Arg.

Optical spectral analysis was performed with a Hewlett-Packard photodiode array spectrophotometer (model HP8452A) equipped with a thermojacketed cell holder and circulating water bath. Addition of compounds was made to sample cuvettes (0.3 mL) with a 10-μL gas-tight syringe to minimize sample dilution. High-spin iNOS samples were first converted to low spin by the addition of up to 1 mM imidazole followed by sequential addition of the L-Arg analogue. Apparent binding constants were determined from the difference spectra of the enzyme−inhibitor complex by fitting the change in absorbance maxima and minima to $y = (aI)/(K_{app} + I)$, where y is the change in absorbance, a is the maximum absorbance change, I is the inhibitor concentration, and K_{app} is the apparent inhibitor dissociation constant. The equilibrium dissociation constant, K_d, was calculated after correction for the presence of imidazole, according to $K_{app} = K_d(1 + I/K_{im})$, where I is the concentration of imidazole and K_{im} is the imidazole dissociation constant. SigmaPlot software (version 4.14, Jandel Scientific) and MacNlin (version 1.0b7, inhouse kinetics program) were used for fitting data, error analysis, and generation of theoretical curves. CO-difference spectra were performed for NADPH-reduced enzyme after purging samples with dry argon followed by carbon monoxide.

RESULTS

Evaluation of L-Arg Analogues with Varying Methylene Spacing. L-2-Amino-4-guanidinobutanoic acid and L-2-amino-3-guanidinopropionic acid have sequentially shorter methylene spacing than L-Arg between the amino acid and the guanidino functional groups (Figure 1). These compounds were evaluated as substrates by measuring the levels of nitrite, a degradation product of NO in solution, after prolonged incubation with the enzyme (Figure 2). L-Arg (K_m = 1.7 μM) (*23*) and L-homoarginine were substrates, but neither of the compounds with shorter methylene chains gave any detectable nitrite. Although L-2-amino-4-guanidinobutanoic acid and L-2-amino-3-guanidinopropionic acid were not utilized by iNOS as substrates, both of these compounds were inhibitors with IC_{50} values of 284.5 ± 42.3

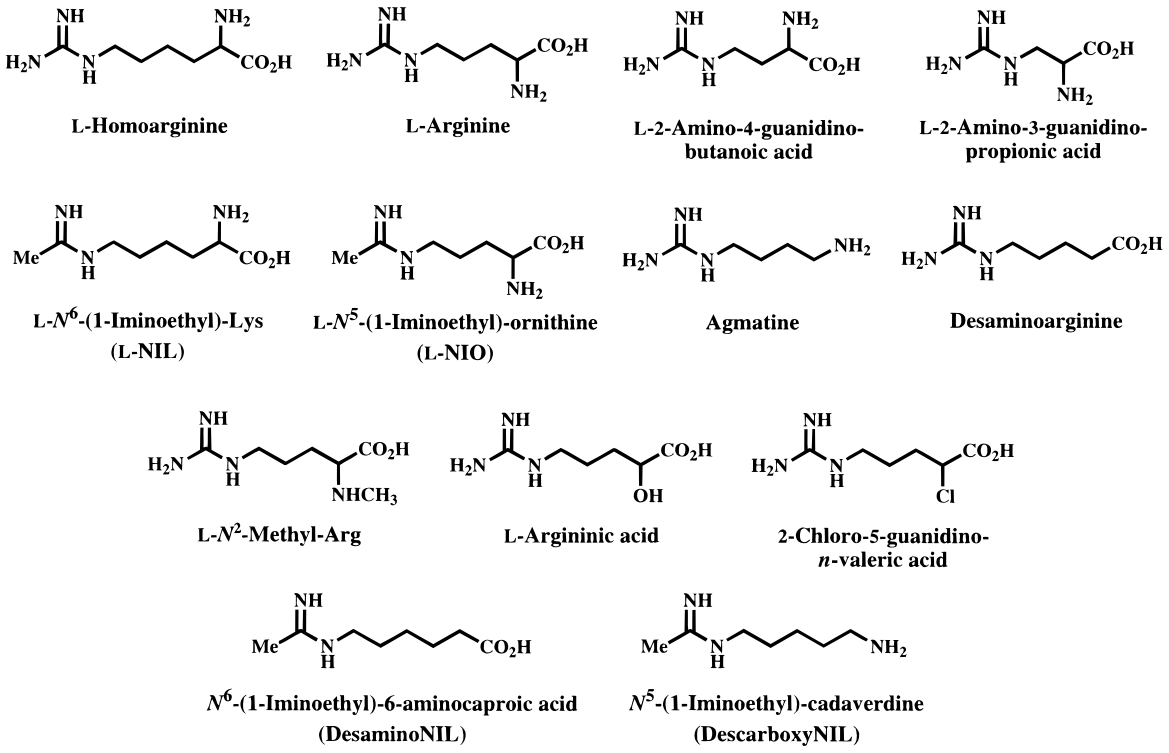

FIGURE 1: Structures of compounds.

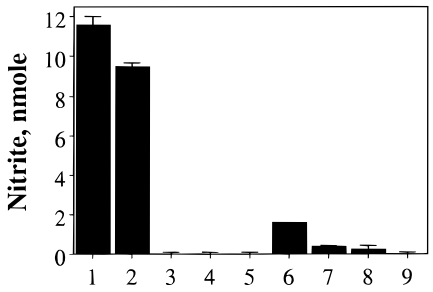

FIGURE 2: Relative nitrite levels for L-Arg and analogues. Compounds **1**−**9** were L-Arg, L-homoarginine, L-2-amino-4-guanidinobutanoic acid, L-2-amino-3-guanidinopropionic acid, desaminoarginine, agmatine, L-argininic acid, L-N^2-methylarginine, and 2-chloro-5-guanidino-*n*-valeric acid. Samples were run in triplicate with standard deviations shown.

and 23.2 ± 2.8 μM, respectively. These results predict that there is a critical spacing between the guanidino and amino acid groups for compounds to be utilized as substrates. Yet, compounds with shorter spacing than L-Arg can still bind to the enzyme, since L-2-amino-4-guanidinobutanoic acid and L-2-amino-3-guanidinopropionic acid were inhibitors of iNOS activity.

L-N^6-(1-Iminoethyl)lysine (L-NIL) and L-N^5-(1-iminoethyl)ornithine (L-NIO) are structural analogues of the NOS substrates L-homoarginine and L-Arg, respectively, but contain an amidino functional group rather than a guanidino group (Figure 1). These compounds are not iNOS substrates but are inhibitors of iNOS activity (*1*, *9*, *24*). IC_{50} values for the inhibition of iNOS by L-NIL and L-NIO were 1.0 ± 0.1 and 1.8 ± 0.3 μM, respectively. Progress curves for the inhibition of iNOS by L-NIL and L-NIO were nonlinear (Figure 3). Table 1 shows the kinetic parameters for the time-dependent inhibition of iNOS by these compounds under subsaturating substrate conditions.

Preincubation studies were used to evaluate the reversible/irreversible nature of the time-dependent enzyme inhibition by L-NIL and L-NIO. Enzyme was preincubated with L-NIL or L-NIO at their respective IC_{50} values in the presence of NADPH and cofactors, but without L-Arg. The solutions were then diluted with a buffer containing 10 μM L-Arg to a volume one-tenth the IC_{50} value of the inhibitor. Enzyme activity could not be recovered after dilution, demonstrating that inhibition of iNOS by L-NIL or L-NIO is irreversible. Kinetic parameters for the inactivation of iNOS by L-NIL were determined by preincubating the enzyme with variable L-NIL concentration for different preincubation times (Figure 4). The kinetic parameters for inactivation of iNOS by L-NIL determined in this way are in good agreement with the values obtained from time courses at subsaturating L-Arg concentration (Table 1). Preliminary data were reported by another laboratory for murine iNOS (m-iNOS) consistent with irreversible inhibition by L-NIL, but no further explanation for this inactivation was provided (*9*). We have further investigated the inactivation of rh-iNOS by L-NIL with respect to cosubstrate requirements.

The role of NADPH in the mechanism of enzyme inactivation by L-NIL and L-NIO was evaluated by preincubating the enzyme with inhibitor in the presence and absence of NADPH. In the absence of NADPH, the inhibition by L-NIL (Figure 5) was reversed after dilution with nearly complete recovery of enzyme activity. However, when NADPH was present in the preincubation mixture, irreversible loss of activity was observed (Figure 5A). The same NADPH-dependent inactivation of iNOS was also observed for L-NIO. The oxidized forms of the nicotinamides, $NADP^+$ and NAD^+, did not support enzyme inactivation by L-NIL, but NADH was able to substitute for NADPH (Figure 5B). Although NADH was not a suitable cosubstrate for NO production by m-iNOS (*8*), we have

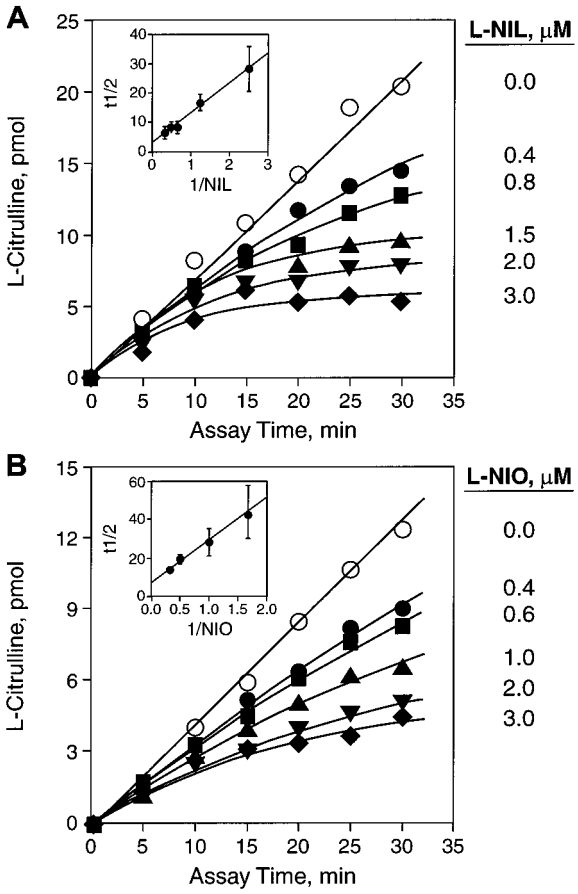

FIGURE 3: Time-dependent inactivation of iNOS: A, time courses for inhibition of iNOS by L-NIL; B, time courses for inhibition of iNOS by L-NIO. Time courses for L-citrulline production were measured at subsaturating levels of substrate (1 μM L-Arg) and variable inhibitor concentration. Inserts, double-reciprocal replots for the half-life, $t_{1/2}$, versus reciprocal inhibitor concentration.

Table 1: Kinetic Parameters for Inactivation of INOS

parameters	time course[a]	preincubation[b]
L-NIL		
k_{inact}, min^{-1}	0.22 ± 0.08	0.24 ± 0.05
K_I, μM	3.2 ± 0.8	4.1 ± 0.8
k_{inact}/K_I, $M^{-1}min^{-1}$	$(6.8 \pm 3.0) \times 10^4$	$(5.9 \pm 1.7) \times 10^4$
L-NIO		
k_{inact}, min^{-1}	0.12 ± 0.03	
K_I, μM	3.9 ± 0.7	
k_{inact}/K_I, $M^{-1}min^{-1}$	$(3.0 \pm 0.8) \times 10^4$	
descarboxy-NIL		
k_{inact}, min^{-1}	0.09 ± 0.03	
K_I, μM	14.8 ± 5.9	
k_{inact}/K_I, $M^{-1}min^{-1}$	$(5.9 \pm 3.1) \times 10^3$	
L-NMA[c]		
k_{inact}, min^{-1}		0.05
K_I, μM		4.2
k_{inact}/K_I, M^{-1} min^{-1}		1.2×10^4

[a] Values determined from time courses of iNOS inhibition at subsaturating L-Arg. [b] Values determined from inactivation replots of remaining iNOS activity at saturating L-Arg after preincubation with variable inhibitor in the absence of L-Arg. [c] Inactivation data from ref *7*.

found that NADH can be utilized by rh-iNOS for L-Arg to L-citrulline conversion, but the initial velocity of the reaction is 18% of the rate with NADPH.

The NADPH-dependent loss of iNOS activity was also examined under anaerobic conditions to evaluate the role of oxygen in the mechanism of enzyme inactivation by L-NIL. Preincubation of enzyme with L-NIL and NADPH was performed under inert argon atmosphere where iNOS activity was reduced to below 9% of its activity in the presence of dioxygen. Preincubation of enzyme with 4 μM L-NIL and 0.5 mM NADPH caused irreversible loss of enzyme activity under aerobic conditions ($24.0 \pm 7.1\%$ residual activity, $n = 4$), but under anaerobic conditions most of the enzyme activity could be recovered after dilution ($83.4 \pm 13.5\%$ residual activity, $n = 7$). Therefore, the inactivation of

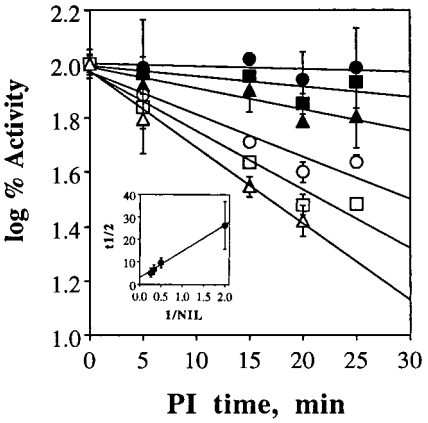

FIGURE 4: Preincubation studies of iNOS with L-NIL. Inactivation plot of the log percent residual iNOS activity after preincubation with variable L-NIL concentration as a function of preincubation time. Enzyme and L-NIL were preincubated without substrate but with NADPH for a set preincubation time and then diluted into assay buffer containing 10 μM L-Arg. The concentrations of L-NIL in the preincubations were 0.0 μM (●), 0.5 μM (■), 1.0 μM (▲), 2.0 μM (○), 3.0 μM (□), and 4.0 μM (△). Data points were from duplicate determinations. Insert, replot for the half-life, $t_{1/2}$, versus reciprocal inhibitor concentration.

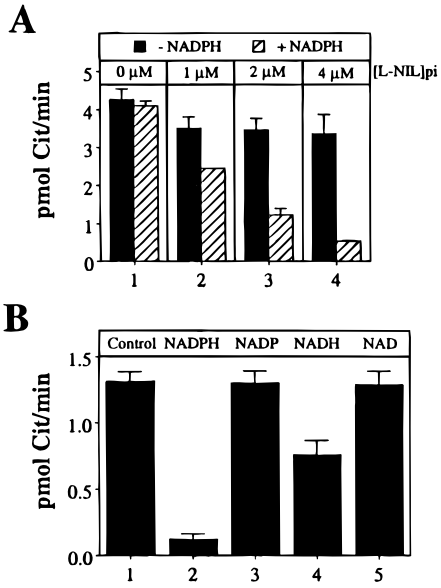

FIGURE 5: Nicotinamide cofactor requirements for the irreversible inactivation of iNOS by L-NIL. A, NADPH requirement for enzyme inactivation by L-NIL. Bar graph shows the residual enzyme activity after preincubation with L-NIL and ±0.5 mM NADPH followed by dilution into assay buffer. B, Comparison of nicotinamide cofactors for the inactivation of iNOS by L-NIL. Bar graph shows the residual enzyme activity after preincubation with 0.1 mM reduced or oxidized form of cofactors with 4 μM L-NIL followed by dilution into assay buffer containing 0.5 mM NADPH. Data points were determined in triplicate with error bars indicating standard deviations.

iNOS by L-NIL is dependent on both NADPH and dioxygen.

Radiolabeled inhibitor, DL-[4,5-^{3}H]NIL, was used as a probe to determine if the mechanism of inactivation of iNOS by L-NIL involves covalent modification of the protein. After incubation of the enzyme with [^{3}H]NIL followed by exhaustive dialysis, only about 0.2% of enzyme was labeled by the inhibitor. This means either that the mechanism of inactivation does not include covalent modification of the enzyme or that radiolabel can dissociate from the enzyme. It is possible that one of the essential cofactors such as tetrahydrobiopterin are modified during the inactivation and might be removed by exhaustive dialysis. However, we have not been able to detect any low-molecular-weight radiolabeled byproducts of the inactivation by HPLC analysis.

The reversible binding of L-NIL to iNOS in the absence of NADPH was further examined by optical spectroscopy. The addition of increasing amounts of L-NIL to an iNOS−imidazole low-spin complex converted the heme to high-spin, type I spectrum (Figure 6A). The induced spectral change upon binding of L-NIL is similar to that observed for L-Arg (*25*). A K_d was determined for L-NIL of 4.6 ± 0.6 μM (Figure 6A, insert) which is in reasonable agreement with the K_I of 3.2 ± 0.8 μM determined by the enzyme inactivation studies (Table 1). L-NIL also reversed the imidazole-induced low-spin heme under anaerobic conditions with a type I difference spectrum and a K_d of 9.1 ± 2.3 μM. Although the inactivation of iNOS by L-NIL requires NADPH and dioxygen, these spectral studies show that L-NIL can bind reversibly to iNOS in their absence. Upon the addition of NADPH to the L-NIL−enzyme complex, the type I band was unchanged. Incubation of enzyme with L-NIL and NADPH (15 °C, 2−3 h) caused no significant decrease in the total heme absorbance of iNOS. In fact, enzyme incubated with 20 μM L-NIL and 0.1 mM NADPH for 30 min still gave a characteristic P450 CO-difference spectrum (Figure 6B) despite being 70% inactivated. P420 formation was not observed after inactivation of rh-iNOS by L-NIL, although this has been proposed as a mechanism of inactivation of tetrahydrobiopterin-depleted neuronal NOS (*26*).

Evaluation of 2-Substituted Analogues of L-Arg and L-NIL. The role of the amino acid portion of structural analogues of L-Arg and L-NIL in binding and in the catalytic mechanism was further evaluated. Desaminoarginine, agmatine, L-argininic acid, L-N^2-methylarginine, and 2-chloro-5-guanidino-*n*-valeric acid (Figure 1) were examined as potential iNOS substrates. These compounds differ from L-Arg solely by the substitution at the 2-position. Desaminoarginine and 2-chloro-5-guanidino-*n*-valeric acid were not substrates for iNOS. Nitrite was detected for incubation of iNOS with agmatine, L-argininic acid, and L-N^2-methylarginine (Figure 2). This suggests that L-Arg analogues with an amino, hydroxyl, or secondary amino group may be substrates for iNOS. However, the levels of nitrite produced by these compounds were quite low compared to that of L-Arg over the time course of the reaction. (For the commercial preparations of agmatine and L-argininic acid, a small contaminating amount of L-Arg might account for the observed nitrite.) When assayed as inhibitors of L-citrulline production, desaminoarginine (IC_{50} = 3.2 ± 0.5 μM) was the most potent of the L-Arg analogues followed by agmatine

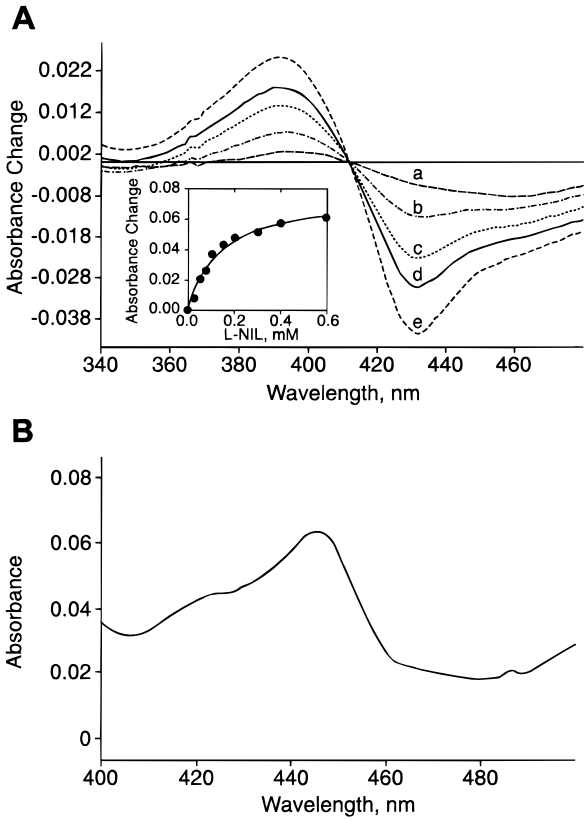

FIGURE 6: Optical spectra of iNOS−L-NIL complexes. A, Type I difference spectra for addition of L-NIL to a iNOS−imidazole complex. Enzyme (0.2 mg) was equilibrated at 15 °C in buffer (0.3 mL) containing 20 mM TES (pH 7.4), 0.2 M NaCl, 1 mM CHAPS, 0.1 mM BH_4, 0.1 mM DTT, and 1 mM imidazole. Sequential additions of L-NIL (representative spectra, a−e: 0.03, 0.05, 0.10, 0.20, 0.98 mM) to the iNOS−imidazole complex caused a new absorbance maximum at 394 nm, an absorbance minimum at 432 nm, and an isobestic point at 412 nm. Insert, Determination of L-NIL spectral binding constant from a plot of the change in absorbance ($A_{max-min}$) versus inhibitor concentration. K_d was determined after correction for the initial presence of imidazole (1 mM). B, CO-difference spectra of NADPH-reduced iNOS−L-NIL complex with absorbance maximum at 444 nm. Enzyme (0.3 mg) was equilibrated at 25 °C in buffer (0.6 mL) containing 0.1 M TES (pH 7.5), 0.1 mM NADPH, 0.1 mM BH_4, and 20 μM L-NIL. The sample was incubated aerobically for 30 min followed by sparging with argon and carbon monoxide.

(IC_{50} = 98.5 ± 14.5 μM), L-N^2-methylarginine (IC_{50} = 106.2 ± 13.5 μM), and L-argininic acid (IC_{50} = 388.0 ± 46.5 μM). 2-Chloro-5-guanidino-*n*-valeric acid was a poor inhibitor of the enzyme with an IC_{50} value of 14.4 ± 1.7 mM. The reversible binding of desaminoarginine, agmatine, and L-argininic acid was also examined by optical spectroscopy. Each of these compounds gave a type I difference spectrum with iNOS (Figure 7) similar to L-Arg (*25*) and L-NIL with K_d values of 3.8 ± 0.1, 239.1 ± 25.3, and 144.8 ± 6.8 μM, respectively. These results demonstrate that L-Arg analogues without a 2-amino or 2-carboxy group bind in a fashion similar to that of the enzyme but that the 2-amino group (or a suitable substitution) is required for catalysis.

If the 2-amino group is essential for substrate catalysis, perhaps this group might also be important for the mechanism-based enzyme inactivation mechanism of L-NIL. We therefore tested N^6-(1-iminoethyl)-6-aminocaproic acid and N^5-(1-iminoethyl)cadaverdine, the corresponding desamino and descarboxy analogues of L-NIL (Figure 1), for their

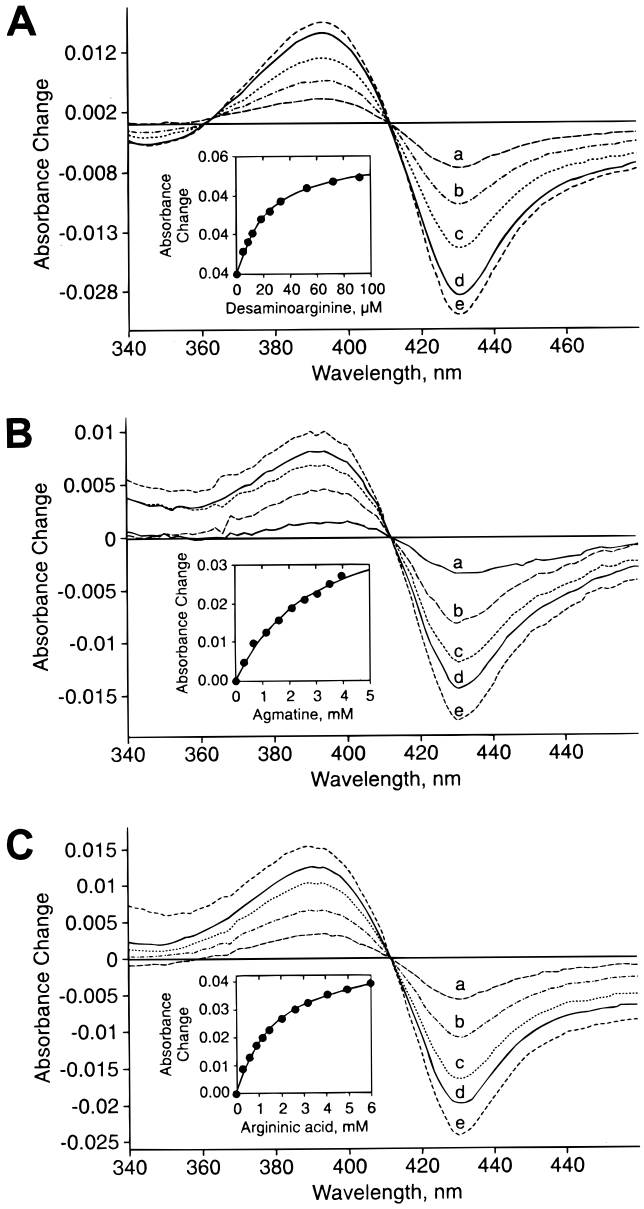

FIGURE 7: Optical absorbance spectra of iNOS−L-Arg analogue complexes. Enzyme (60 μg) was equilibrated at 15 °C in buffer (0.3 mL) containing 20 mM TES (pH 7.4), 1 mM CHAPS, 0.1 mM BH_4, 0.1 mM DTT, and 0.2 M NaCl. Imidazole (0.1 mM) was added to form low-spin heme. The low-spin heme was reversed to high-spin heme (type I) by the addition of desaminoarginine, agmatine, or L-argininic acid. A, Representative spectra, a−e: [desaminoarginine] = 5, 12, 25, 52, and 92 μM with an absorbance maximum at 392 nm, an absorbance minimum at 430 nm, and an isobestic point at 412 nm. B, Representative spectra, a−e: [agmatine] = 0.3, 1.1, 2.1, 3.1, and 4.0 mM with an absorbance maximum at 392 nm, an absorbance minimum at 430 nm, and an isobestic point at 412 nm. C, Representative spectra, a−e: [L-argininic acid] = 0.3, 0.9, 2.1, 3.2, and 6.1 mM with an absorbance maximum at 390 nm, an absorbance minimum at 430 nm, and an isobestic point at 412 nm. Inserts, Determination of binding constants from a plot of the change in absorbance ($A_{max-min}$) versus inhibitor concentration.

inhibitory activity toward iNOS. Both desamino-NIL and descarboxy-NIL were inhibitors of iNOS with IC_{50} values of 68.6 ± 9.8 and 8.3 ± 0.2 μM, respectively. Since L-NIL is a mechanism-based enzyme inactivator, desamino-NIL and descarboxy-NIL were also examined as potential inactivators of iNOS. When enzyme was preincubated with either 200 μM desamino-NIL or 12 μM descarboxy-NIL in the absence

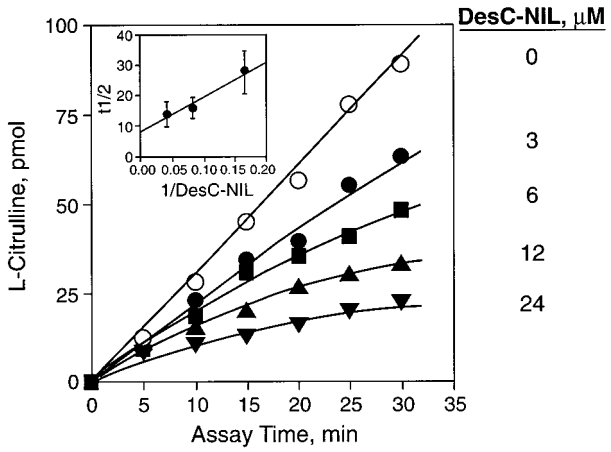

FIGURE 8: Time-dependent inhibition of iNOS by descarboxy-NIL. Time courses for L-citrulline production were measured at subsaturating levels of substrate (1 μM L-Arg) and variable inhibitor concentration as shown. Experiments were performed in duplicate with representative curves shown.

of NADPH, all of the enzyme activity could be recovered after dilution. For desamino-NIL, enzyme activity could also be recovered by dilution when preincubated with NADPH. Unlike desamino-NIL, however, descarboxy-NIL irreversibly inhibited iNOS activity when preincubated with NADPH. Progress curves for the inhibition of iNOS by descarboxy-NIL also showed time-dependent inhibition (Figure 8) with kinetic parameters for inactivation similar to those obtained with L-NIL and L-NIO (Table 1). It appears then that the 2-amino group of L-NIL is required for enzyme inactivation. These results provide further support for a mechanistic role for the 2-amino group.

DISCUSSION

The structure−activity studies described herein have further defined structural elements required for rh-iNOS substrates, reversible inhibitors, and mechanism-based enzyme inactivators. Our results demonstrate that the amino acid portions of the substrate molecule and amidino amino acid inhibitors are not required for binding to the enzyme but the 2-amino group has an important role in catalysis. The amino group of agmatine and descarboxy-NIL, the 2-hydroxy of L-argininic acid, and the secondary 2-amino group of L-N^2-methylarginine are all capable of hydrogen bonding, but desaminoarginine, desamino-NIL, and 2-chloro-5-guanidino-*n*-valeric acid do not have corresponding hydrogen-bonding functional groups. We propose that there is a hydrogen-bonding interaction at this 2-position between the substrate or inhibitor and enzyme which is required for catalysis. This interaction may be important for the productive binding of the substrate or perhaps is involved in an essential conformational change in the active site.

A previous study using a panel of L-Arg analogues found that the binding of compounds within the heme domain of m-iNOS was essentially independent of the amino acid group (*27*). In that study, a type I spectral change was not observed for the binding of L-argininic acid to m-iNOS, nor was agmatine found to be a substrate for m-iNOS (*27*). These differences may be attributable to species variability between human and murine iNOS.

The NADPH-dependent inactivation of rh-iNOS by L-NIL and its analogues is similar in part to the NADPH-dependent

mechanism of inactivation of m-iNOS by NMA (*7*, *8*, *28*). The kinetic mechanism of inactivation of rh-iNOS by L-NIL and L-NIO has similar inactivation parameters as those reported for m-iNOS with NMA (see Table 1) (*7*, *8*). However, the inactivation of m-iNOS by NMA involves multiple chemical mechanisms which do not appear to be involved with the inactivation of rh-iNOS by L-NIL. Inactivation of m-iNOS by NMA (*7*, *8*, *28*) is characterized by the following: (i) NADPH-dependent hydroxylation of NMA forming formaldehyde and L-Arg, (ii) the partial, covalent radiolabeling of the protein, and (iii) the substantial loss of enzyme-bound heme. Unlike the inactivation of m-iNOS by NMA, a mechanism for formaldehyde release cannot be written for L-NIL or L-NIO since C-hydroxylation would require carbon−carbon bond cleavage. Perhaps N-hydroxylation of the inhibitor is occurring but without subsequent formation of NO. The resulting N-hydroxylation product may be further oxidized to give another nitrogen species that may be responsible for the observed enzyme inactivation. Alternatively, the binding of these inactivators within the heme domain of iNOS could induce the formation of a reactive oxygen species which may irreversibly modify the enzyme or prosthetic groups and would account for the dioxygen requirement for the inactivation. Marletta and coworkers (*28*) found that [2,3-^{3}H]NMA labeled the protein up to 11% which is much greater than the 0.2% observed here for [^{3}H]NIL. They also found a significant loss of the heme following inactivation of m-iNOS by NMA (*28*). However, there was little loss of rh-iNOS heme after incubation with L-NIL and NADPH as judged by the CO-reduced difference spectrum.

Overall we conclude that in addition to the previously demonstrated selectivity for L-amino acids, requirements for iNOS substrates include (i) a minimum three methylene spacing between the guanidino group and the amino acid portion of the substrate and (ii) the presence of a hydrogen-bonding group such as an amino or hydroxyl at the 2-position. Furthermore, we extend the catalytic requirement for the 2-amino group of L-NIL analogues which act as mechanism-based inactivators of iNOS. What is still needed is to ascertain what specific interactions are involved in triggering the catalytic steps leading to either substrate turnover or mechanism-based enzyme inactivation. It will also be interesting to see if these interactions are also important for the catalytic mechanisms of other NOS isoforms.

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