Analysis of Substrate-Induced Electronic, Catalytic, and Structural Changes in Inducible NO Synthase†

Nicolas Sennequier‡ and Dennis J. Stuehr*,‡,§

Department of Immunology, The Cleveland Clinic, Cleveland, Ohio, 44195, and Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio, 44106

Received December 1, 1995; Revised Manuscript Received March 5, 1996⊗

ABSTRACT: Inducible nitric oxide synthase (iNOS) catalyzes the NADPH-dependent formation of nitric oxide (NO) and citrulline from L-arginine and O_2. In addition to serving as substrate, L-arginine alters the enzyme's heme iron spin equilibrium, increases its NADPH oxidation, and promotes assembly of active dimeric iNOS from inactive monomers. To understand what structural aspects of L-arginine are important for causing these effects, we have studied the interactions of iNOS with several L-arginine and guanidine analogs. Very few analogs supported NO synthesis even when bound to iNOS at saturating or near-saturating levels. In contrast, almost all analogs shifted the heme iron spin equilibrium and either increased or decreased NADPH oxidation by iNOS. The guanidine analogs displayed the same pattern of effects as their amino acid counterparts but exhibited a lower affinity except for analogs containing *S*-alkylisothiourea or aminoguanidine groups. Most analogs also promoted iNOS dimerization, with hydroxyguanidine and *S*-ethylisothiourea promoting more dimerization than L-arginine itself. Although the analog concentrations required to promote dimerization of monomers were somewhat higher than those required for binding to dimeric iNOS, they followed the same rank order. The degree of dimerization promoted by each analog did not correlate to its binding affinity, its causing a high- or low-spin shift in heme iron spin state, or to its increasing or decreasing NADPH oxidation. Together, we conclude that the enzyme's high degree of substrate specificity only applies to NO synthesis, in that a number of "inactive" structural analogs still bind to iNOS and affect its heme chemistry and structure in the absence of supporting NO synthesis. These latter affects are mediated through binding of the guanidinium portion of L-arginine and its analogs to a single site within iNOS and are relatively independent of the amino acid portion of the molecule.

Nitric oxide (NO)[1] biosynthesis has important roles in mammalian physiology and pathology [reviewed by Bredt and Snyder (1994), Nathan and Xie (1994), Schmidt and Walter (1994), and Stamler (1994)]. NO is generated by three distinct NO synthases (NOSs) (Marletta, 1994; Griffith & Stuehr, 1995). The two NOS isoforms that are expressed in neurons or endothelium synthesize NO in response to Ca^{2+}-promoted calmodulin binding and thus serve in signal transduction cascades by coupling NO synthesis to transient increases in intracellular Ca^{2+} (Bredt & Snyder, 1994). A third NOS isoform (iNOS) is expressed in cells only following exposure to immunostimulating cytokines or bacterial products (Kroncke *et al*., 1995). It synthesizes NO independently of the intracellular Ca^{2+} concentration, due to tight binding of calmodulin to its recognition site (Cho *et al*., 1992). Many cell types can express iNOS, and it appears to function in host defense against microbial and viral pathogens as well as participate in inflammatory diseases including rheumatoid arthritis (McCartney-Francis *et al*., 1993), multiple sclerosis (Boullerne *et al*., 1995), and hypotension during septic shock (Radomski *et al*., 1990).

All three NOS appear to be active as homodimers, with each subunit exhibiting a bi-domain structure comprised of an N-terminal oxygenase domain linked to a C-terminal reductase domain (Sheta *et al*., 1994; Baek *et al*., 1993; Ghosh & Stuehr, 1995; Abu-Soud & Stuehr, 1994b; McMillan & Masters, 1995). The reductase domain contains NADPH, FMN, FAD, and calmodulin binding modules, and functions to transfer electrons from NADPH to the oxygenase domain, which contains the binding sites for iron protoporphyrin IX (heme), H_4biopterin, and substrate (L-arginine). Calmodulin controls communication between the two domains in that its binding triggers flavin-to-heme electron transfer (Abu-Soud & Stuehr, 1993). Heme iron reduction is thought to lead to oxygen activation and synthesis of NO and citrulline from L-arginine in a stepwise reaction that requires 1.5 equiv of NADPH per mole of NO formed (Stuehr *et al*., 1991; Klatt *et al*., 1993; Marletta, 1994; Griffith & Stuehr, 1995).

† This work was supported by National Institutes of Health Grant CA53914. D.J.S. is an Established Investigator of the American Heart Association.

* Corresponding author. Tel: (216) 445-6950. FAX: (216) 444-9329. E.mail: stuehrd@cesmtp.ccf.org.

‡ The Cleveland Clinic.

§ Case Western Reserve University.

⊗ Abstract published in *Advance ACS Abstracts,* April 15, 1996.

[1] Abbreviations: AGPA, L-α-amino-β-guanidinopropionic acid; arginiinic acid, L-α-hydroxy-δ-guanidinovaleric acid; BSA, bovine serum albumin; CaM, calmodulin; DTT, dithiothreitol; EPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; H_4biopterin, (6*R*,6*S*)-2-amino-4-hydroxy-6-(L-*erythro*-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropteridine; HbO_2, oxyhemoglobin; NAA, N^{ω}-amino-L-arginine; NAME, N^{ω}-nitro-L-arginine methyl ester; NMA, N^{ω}-methyl-L-arginine; NOHarg, N^{ω}-hydroxy-L-arginine; NO, nitric oxide; NOS; nitric oxide synthase; SEITU, *S*-ethylisothiourea; SMTC, *S*-methyl-L-thiocitrulline; thiocitrulline, L-α-amino-δ-thioureidovaleric acid.

S0006-2960(95)02844-3 CCC: $12.00

The NOS heme iron is axially coordinated to a cysteine thiolate that is located in the oxygenase domain (White & Marletta, 1992; Stuehr & Ikeda-Saito, 1992; McMillan & Masters, 1992, 1995). In the H_4biopterin-saturated enzyme, the heme iron is predominantly five-coordinate, with an overall ligand environment similar but not identical to the heme iron in the cytochromes P450 (Stuehr & Ikeda-Saito, 1992; McMillan *et al*., 1992; Sono *et al*., 1995; Wang *et al*., 1993, 1994, 1995). L-Arginine binds close enough to the NOS heme iron to cause a shift in the iron spin state equilibrium toward high-spin (McMillan & Masters, 1993). Bound L-arginine also sterically interacts with heme iron ligands such as NO and CO and reduces the access of CO or cyanide to the heme iron (Matsuoka *et al*., 1994; Wang *et al*., 1993, 1994). Only three compounds are known to act as alternative substrates: The reaction intermediate N^{ω}-hydroxy-L-arginine (NOHarg) (Stuehr *et al*., 1991; Klatt *et al*., 1993; Pufahl *et al*., 1992), N^{ω}-methyl-L-arginine (NMA) (Olken & Marletta, 1993; Feldman *et al*., 1993; Klatt *et al*., 1994), and homoarginine (Yokoi *et al*., 1995). A variety of related compounds can also bind within the substrate binding pocket and act as competitive inhibitors, including N^{ω}-substituted arginines (Furfine *et al*., 1993, Komori *et al*., 1994) and guanidines (Wolff & Lubeskie, 1995), thioureas (Narayanan & Griffith, 1993; Abu-Soud *et al*., 1994a; Narayanan *et al*., 1995), and isothioureas (Furfine *et al*., 1994; Garvey *et al*., 1994; Nakane *et al*., 1995).

L-Arginine binding alters electron flux through NOS as reflected by a change in NOS oxygen consumption (Pou *et al*., 1992; Heinzel *et al*., 1992) or NADPH oxidation (Abu-Soud & Stuehr, 1993; Abu-Soud *et al*., 1994a). Analogs of L-arginine known to up- or down-regulate NADPH oxidation by NOS include NOHarg, NMA, *S*-methylisothiourea, N^{ω}-nitro-L-arginine, N^{ω}-nitro-L-arginine methyl ester (NAME), and thiocitrulline (Heinzel *et al*., 1992; Abu-Soud *et al*., 1994; Furfine *et al*., 1994; Garvey *et al*., 1994). Thiocitrulline and NAME inhibit NADPH oxidation apparently by decreasing the reduction potential of the NOS heme iron (Abu-Soud *et al*., 1994a). In general, positive or negative changes in NADPH oxidation rates can be used to estimate binding constants for analogs that occupy the substrate binding site but do not serve as substrates for NO synthesis (Abu-Soud *et al*., 1994a).

In addition to serving as the substrate, L-arginine is required along with H_4biopterin and heme for iNOS subunits to assemble into an active dimeric enzyme (Baek *et al*., 1993; Abu-Soud *et al*., 1995). The ability of L-arginine to promote subunit dimerization is stereospecific (D-arginine is ineffective) but apparently not exclusive, because the reaction intermediate NOHarg can substitute for L-arginine in promoting dimer assembly (Baek *et al*., 1993). Since iNOS is only active in dimeric form, dimer assembly has been proposed as a possible point for pharmacologic intervention. However, it is unclear what structural features of L-arginine are important for promoting dimerization, and if those same features also control the binding of L-arginine to the dimer, its functioning as a substrate, or its altering electron flux through iNOS.

To address these issues, we have examined several L-arginine, guanidine, and thiourea analogs regarding their ability to bind to dimeric iNOS, shift the spin state equilibrium of the heme iron, act as a substrate, up- or down-regulate NADPH oxidation, and promote subunit dimerization. The results identify structural features that are important for each of these functions, and provide a unified understanding of L-arginine's interactions with NOS.

MATERIALS AND METHODS

Materials. Thiocitrulline and *S*-methyl-L-thiocitrulline (SMTC) were a kind gift from Dr. Paul L. Feldman, Glaxo Research Institute, Research Triangle Park, NC. N^{ω}-amino-L-arginine (NAA) was a kind gift from Dr. Owen W. Griffith, Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI. Ultrapure urea was obtained from Bio-Rad. L-α-Amino-β-guanidinopropionic acid (AGPA), agmatine, citrulline, homoarginine, argininic acid, aminoguanidine, ornithine, NAME, L-N^{ω}-methylarginine (NMA), imidazole, L-arginine, and D-arginine were purchased from Sigma Chemical. Nitroguanidine, guanidine, methylguanidine, *S*-ethylisothiourea (SEITU), and thiourea were purchased from Aldrich Chemical, St. Louis, MO. N^{ω}-Hydroxy-L-arginine (NOHarg) was purchased from Alexis, San Diego, CA. Hydroxyguanidine was purchased from Pfaltz and Bauer. Pefabloc proteinase inhibitor was purchased from Boeringer Mannheim GmBH, Germany.

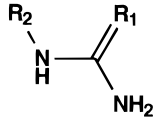
Dimeric iNOS was purified from supernatants of interferon-γ- and *Escherichia coli* lipopolysaccharide-activated RAW 264.7 cells by a two column procedure using a Pharmacia fast protein liquid chromatograph as described in Baek *et al*. (1993).

NO Synthesis Assay. NO synthesis by dimeric iNOS was assayed using the oxyhemoglobin spectrophotometric assay (Feelisch & Noack, 1987). An aliquot of iNOS (to give final [iNOS] 0.01−0.02 μM) was transferred to a cuvette containing 40 mM EPPS buffer, pH 7.9, supplemented with 5−15 μM HbO_2, 0.3 mM DTT, 2 mM arginine, 0.2 mM NADPH, 4 μM each of FAD, FMN, and H_4biopterin, 10 units of catalase/mL, 10 units of SOD/mL, 0.1 mg of BSA/mL, 3 ng of aprotinin/mL, 0.7 μg each of leupeptin and pepstatin A/mL, and 16 μg of Pefabloc/mL to give a final volume of 0.7 mL. The control cuvette contains everything except iNOS and NADPH. The NO-mediated conversion of oxyhemoglobin to methemoglobin was monitored at 37 °C over time as an increase in absorbance at 401 nm and quantitated using an extinction coefficient of 38 mM^{-1} cm^{-1}.

NADPH Oxidase Activity. The rate of iNOS NADPH oxidation was determined spectrophotometrically by monitoring the decrease in absorbance at 340 nm over time, using an extinction coefficient of 6.22 mM^{-1} cm^{-1}. Assays were carried out at 37 °C in cuvettes containing 0.7 mL of 40 mM EPPS buffer, pH 7.9, supplemented with 0.3 mM DTT, 2 mM L-arginine, 4 μM each of FAD, FMN, and H_4biopterin, 10 units of catalase/mL, 10 units of SOD/mL, 0.1 mM NADPH, 3 ng of aprotinin/mL, 0.7 μg each of leupeptin and pepstatin A/mL, and 16 μg of pefabloc/mL. In some cases, the reactions contained either no substrate or individual substrate analogs in place of L-arginine. Reactions were initiated by adding ∼0.5−10 μg of iNOS. When performed in a 96-well microplate, the assay was run in triplicate. The final volume of each well was 150 μL, and each contained quantities of iNOS in proportion to the cuvette assay. The reaction was started by addition of NADPH, and the rate of absorbance change at 340 nm was determined using a Molecular Dynamics kinetic plate reader.

In all cases, the rate of non-enzymatic NADPH oxidation was subtracted from the rates measured in the presence of

Table 1: Analogs Used in This Study



R_1	R_2	name	R_2	name
NH		arginine		guanidine
N-OH		NOHarg		hydroxyguanidine
$N\text{-}CH_3$		NMA		methylguanidine
$N\text{-}NH_2$	$^+H_3N\text{—}CH(CH_2)_3\text{—}COO^-$	NAA		aminoguanidine
$N\text{-}NO_2$		NNA	H	nitroguanidine
O		citrulline		urea
S		thiocitrulline		thiourea
$S\text{-}CH_3$		SMTC		SMITU
$S\text{-}CH_2\text{-}CH_3$				SEITU
$N\text{-}NO_2$	$^+H_3N\text{—}CH(CH_2)_3\text{—}COOMe$	NAME		
	$^+H_3N\text{—}CH(CH_2)_4\text{—}COO^-$	homoarginine		
NH	$^+H_3N\text{—}CHCH_2\text{—}COO^-$	AGPA		
	$HO\text{—}CH(CH_2)_3\text{—}COO^-$	argininic acid		
	$^+H_3N\text{—}CH(CH_2)_3\text{—}$	agmatine		

iNOS and the EC_{50} values for L-arginine or analogs were defined as the concentrations yielding half the maximal effect. For analogs that did not saturate within the concentration range tested, the effect obtained at the highest concentration was considered its maximal effect.

Cytochrome c Reductase Activity. The reduction of cytochrome *c* by iNOS was carried out in a 96-well microplate as previously described (Baek *et al*., 1993).

Optical Difference Spectra. Optical spectra were recorded at room temperature on a Hitachi 2110 spectrophotometer. A base line was recorded for 40 mM EPPS buffer (pH 7.9). A 300 μL sample containing 0.5−1 μM iNOS was transferred into a cuvette and a first spectrum recorded. Then 5 μL of each substrate analog solution was added and a second spectrum recorded. The difference spectrum was obtained by substracting the first spectrum from the second, using SpectraCalc software obtained from Galactic Industries. A high-spin shift was denoted by a gain in absorbance at 385 nm and corresponding loss at 420 nm, while a low-spin shift was denoted by the opposite results (McMillan & Masters, 1993).

Formation and Dimerization of iNOS Monomers. Dissociation of dimeric iNOS in urea was carried out as described previously (Abu-Soud *et al*., 1995). The purified iNOS (1−3 μM) was incubated at 4 °C for approximately 1 h in 40 mM EPPS buffer, pH 7.4, containing 3 mM DTT and 2 M urea. It was then dialyzed at 4 °C overnight in 500 mL of 40 mM EPPS, pH 7.7, containing 0.1 M urea, 3 mM DTT, and 10% glycerol.

To generate dimeric iNOS, the dialyzed monomer preparation was incubated at concentrations between 0.4 and 0.8 μM at 37 °C for 30−90 min in 60−280 μL of EPPS buffer, pH 7.9, containing 3 mM DTT, 10 μM H_4biopterin, 40 μM NADPH, 0.1 mg of bovine serum albumin/mL, and 3 mM L-arginine. In some cases, L-arginine was replaced by a substrate analog at the indicated concentration noted in the text.

Gel Filtration Chromatography. To determine the relative amounts of dimeric iNOS and dissociated iNOS subunits present following a dimerization reaction, size exclusion chromatography was carried out at 4 °C using a Pharmacia Superdex 200 column as described in Abu-Soud *et al.* (1995) and Baek *et al*. (1993). The column was equilibrated at 0.5 mL/min with 40 mM EPPS buffer, pH 7.7, containing 2 μM H_4biopterin, 2 mM DTT, and 10% glycerol. Sample injection volume was 100 μL. Protein in the column effluent was detected at 280 nm using a flow-through detector. The molecular weights of the protein peaks were estimated relative to gel filtration molecular weight standards.

RESULTS

iNOS NADPH Oxidation in the Presence of L-Arginine Analogs. Table 1 contains the structures of the analogs used in this study. We first examined each analog regarding its ability to up- or down-regulate NADPH oxidation. Of the nine L-arginine analogs tested, only homoarginine and NOHarg served as substrates for NO synthesis, consistent with previous results (Yokoi *et al*., 1994; Stuehr *et al*., 1991). Five analogs increased iNOS NADPH oxidation relative to substrate-free enzyme (Figure 1, panel A), as occurs with L-arginine (Abu-Soud *et al*., 1994a). The extra methylene group of homoarginine resulted in a reduced affinity toward iNOS compared to L-arginine, as determined by comparing their respective EC_{50} values for increasing NADPH oxidation

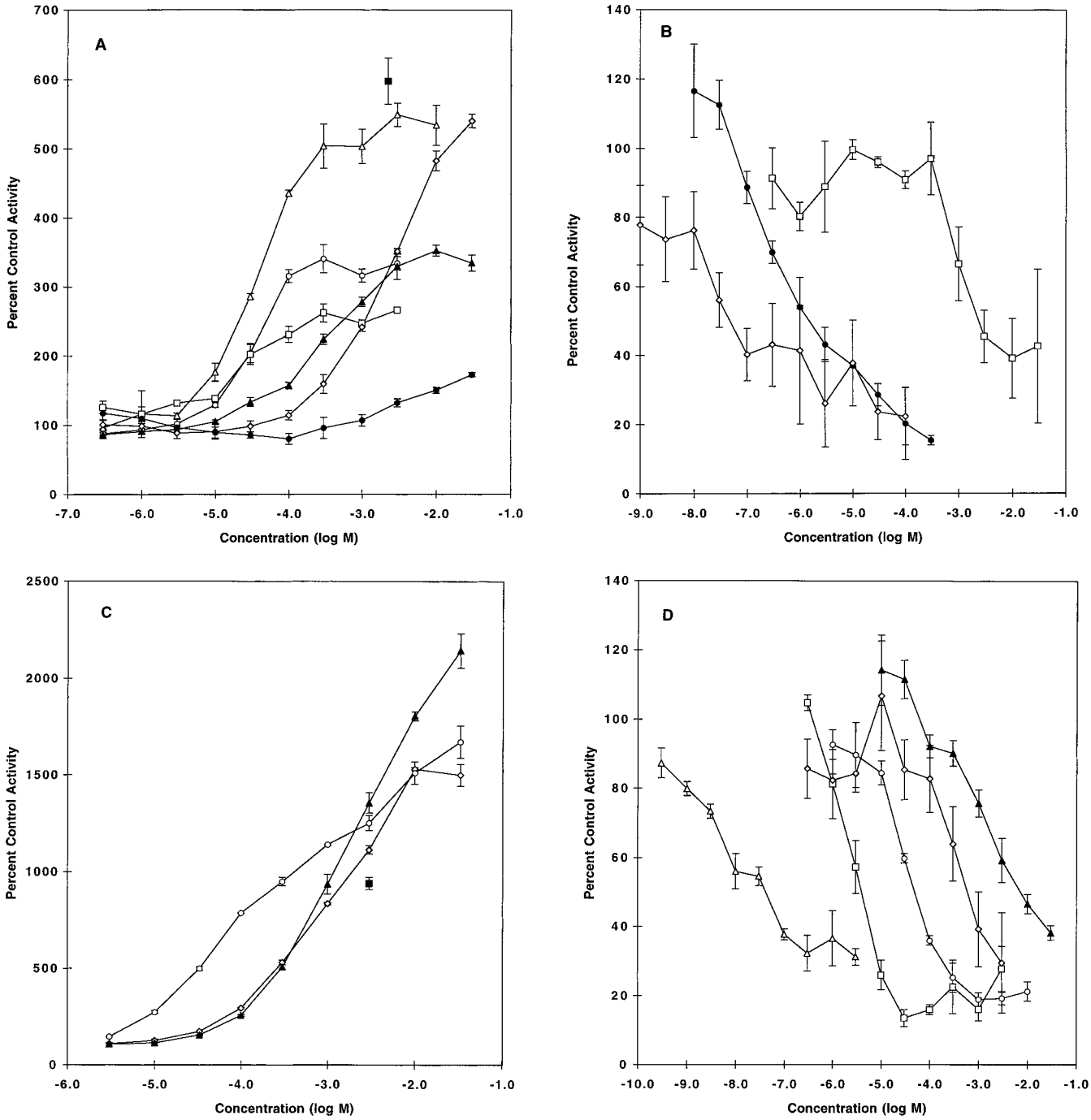


FIGURE 1: NADPH oxidase activity of iNOS as a function of substrate analog concentration. Incubations containing 10 nM of iNOS were prepared in triplicate with decreasing concentrations of each analog. The symbols in panel A represent (■) arginine; (△) homoarginine; (○) NOHarg; (▲) AGPA; (□) agmatine; (◇) argininic acid; and (●) ornithine. In panel B, (◇) SMTC; (●) NAA; and (□) citrulline. In panel C, (■) arginine; (○) methylguanidine; (▲) guanidine; and (◇) hydroxyguanidine. In panel D, (△) SEITU; (□) aminoguanidine; (○) imidazole; (◇) nitroguanidine; and (▲) thiourea. The NADPH oxidation rate by the iNOS preparation in the absence of L-arginine or analogs was 270 ± 25 nmol/min per mg. This value was set as 100%, and the data obtained in the presence of analogs were expressed as a percentage relative to this value.

(Table 2), although homoarginine at saturating concentrations stimulated approximately the same maximal rate of NADPH oxidation as L-arginine. A similar effect was observed with argininic acid, which does not contain the α-amino group, but its effect was subsequently attributed to L-arginine contamination.[2] Shortening the amino acid side chain by two methylene groups (AGPA), removal of the α-carboxyl group (agmatine), or N^{ω}-hydroxylation (NOHarg) decreased the apparent binding affinity of the analog and also reduced the maximum attainable increase in NADPH oxidase activity. Removal of the guanidinium group (ornithine) rendered the analog unable to affect iNOS NADPH oxidation, consistent with the group being important for binding (see below).

As shown in panel B, three amino acid analogs decreased iNOS NADPH oxidation rates relative to substrate-free enzyme. These contained either urea (citrulline), alkyl isothiourea (SMTC), or aminoguanidine (NAA) functional

[2] The increase in iNOS NADPH oxidation observed with argininic acid at millimolar concentrations was likely due to L-arginine contamination, which according to the supplier can reach 0.1%.

Table 2: Substrate Analog Effects on iNOS NADPH Oxidation and Heme Iron Spin State[a]

compound	NADPH oxidase EC_{50} (μM)	effect on NADPH oxidase activity[b]	spin state shift[c]
arginine	12[d]	+	HS
NOHarg	32	+	HS
NMA	17[d]	+	HS
NAA	0.72	−	HS
NAME	32[d]	−	HS
citrulline	1000	−	LS
thiocitrulline	3.5[d,e]	−	LS
SMTC	0.020	−	HS
homoarginine	43	+	HS
AGPA	300	+	HS
argininic acid	2000	+	no effect
agmatine	20	+	HS
ornithine	4000	=	no effect
guanidine	1000	+	HS
hydroxy guanidine	970	+	*f*
methyl guanidine	260	+	HS
aminoguanidine	2.7	−	HS
nitroguanidine	300	−	no effect
thiourea	1700	−	LS
SEITU	0.005	−	HS[g]
imidazole	33	−	LS

[a] Measurements were carried out as described in Materials and Methods. The EC_{50} values were estimated from the curves displayed in Figure 1. The optical difference spectra were taken in solutions receiving 1 mM or less of each analog. [b] +, −, and = mean that the corresponding analog increased, decreased, or did not alter the NADPH oxidase activity of the enzyme compared to the substrate-free control. [c] HS means high-spin shift, and LS means low-spin shift. [d] Abu-Soud *et al*. (1994a). [e] Frey *et al*. (1994). [f] This measure is made impossible by the strong absorbance of hydroxyguanidine in the 350−400 nm region. [g] Garvey *et al*. (1994).

groups in place of the guanidinium group of L-arginine. NAA and SMTC exhibited decreased EC_{50} values relative to L-arginine (Table 2), consistent with their functioning as good competitive inhibitors of NO synthesis (Narayanan & Griffith, 1993; Garvey *et al*., 1994). Citrulline, the enzyme's natural product, also decreased iNOS NADPH oxidation but only when present at millimolar concentrations. As shown in Table 2, analogs that contain an N^{ω}-nitro group (NAME) or thiourea (thiocitrulline) had previously been shown to decrease iNOS NADPH oxidase activity (Abu-Soud *et al*., 1994a).

NADPH Oxidation in the Presence of Guanidine Analogs. To assess the contribution of the functional groups in the amino acids noted above, the corresponding N-substituted guanidines, urea, thiourea, and isothiourea were tested regarding their ability to modulate iNOS NADPH oxidation. *S*-ethylisothiourea (SEITU) was used in place of *S*-methylisothiourea (SMITU), which is not commercially available but has been shown to potently inhibit iNOS NO synthesis (Garvey *et al*., 1994). For additional comparison, we also tested imidazole, which binds directly to the heme iron and inhibits NOS NADPH oxidation and NO synthesis (Wolff & Gribin, 1994).

As shown in Figure 1, panels C and D, and summarized in Table 2, a given guanidine analog either increased or decreased iNOS NADPH oxidase activity in a pattern that matched its identically-substituted amino acid homolog. However, guanidine, hydroxyguanidine, nitroguanidine, methylguanidine, and thiourea all required significantly higher concentrations to affect iNOS NADPH oxidation relative to their amino acid counterparts, whereas aminoguanidine and SEITU were approximately equipotent to their amino acid counterparts. At millimolar concentrations, guanidine, hydroxyguanidine,[3] and methylguanidine each increased iNOS NADPH oxidation to levels that exceeded the maximum rates obtained with either L-arginine or with the corresponding amino acid homologs in panel A. As shown in panel D, each guanidine analog reduced the rate of iNOS NADPH oxidation to levels that approached or matched those observed in the presence of their amino acid homologs. Imidazole also decreased iNOS NADPH oxidation, consistent with its decreasing NADPH oxidation by calmodulin-bound neuronal NOS (Wolff *et al*., 1993). The EC_{50} value for imidazole as estimated from its inhibition of iNOS NADPH oxidation is close to a K_s value of 40 μM which was determined optically (Wolff & Gribin, 1994).

Analog Effect on Spin State Equilibrium. L-Arginine binds above the plane of the NOS heme (Wang *et al*., 1993, 1994) and shifts its heme iron toward a high-spin configuration, as manifested by characteristic changes in the visible spectrum (McMillan & Masters, 1993). The ability of various analogs to perturb the spin equilibrium of the iNOS heme iron is summarized in Table 2. In the absence of L-arginine or analogs, the iNOS used in this study was mostly high-spin. Most of the analogs converted iNOS to a fully high-spin form upon binding. In contrast, citrulline and thiourea caused a shift toward low-spin, consistent with thiocitrulline also causing a partial shift toward low-spin (Frey *et al*., 1994; Abu-Soud *et al*., 1994a). Ornithine or D-arginine did not induce a noticeable change in spin state, consistent with their binding poorly to iNOS.

Analogs as Substrates for NO Synthesis. As noted previously, NOHarg, NMA, and homoarginine are alternative substrates of iNOS. We therefore examined if the other amino acid or guanidine analogs used in the current study could serve as an alternative substrate for NO synthesis. Each analog was tested at a concentration that saturated or nearly saturated the enzyme, as determined by the NADPH oxidation studies. Only argininic acid supported detectable NO synthesis, which was likely due to its containing L-arginine as a contaminant.[2]

Analog Effect on Reductase Activities. Because transfer of NADPH-derived electrons procedes from NADPH through the flavins to the heme iron in NOS, it appeared necessary to examine if the analogs would affect electron transfer events upstream from the heme iron, which can be determined by measuring the rate of iNOS reduction of cytochrome *c*. None of the analogs that increased or decreased iNOS NADPH oxidation affected the cytochrome *c* reductase activity of iNOS (data not shown). This confirms that L-arginine and analogs affect electron transfer via interactions with the heme iron (Abu-Soud *et al*., 1994a).

Ability of Analogs to Support iNOS Dimer Assembly. Previous work had shown that L-arginine and NOHarg could support iNOS dimer assembly, which was associated with a proportional recovery of NO synthesis activity (Baek *et al*.,

[3] At millimolar concentrations, hydroxyguanidine itself promoted some NADPH oxidation. Therefore, the rate of non-enzymatic NADPH oxidation attributed to hydroxyguanidine was substracted from the values obtained in the presence of iNOS.

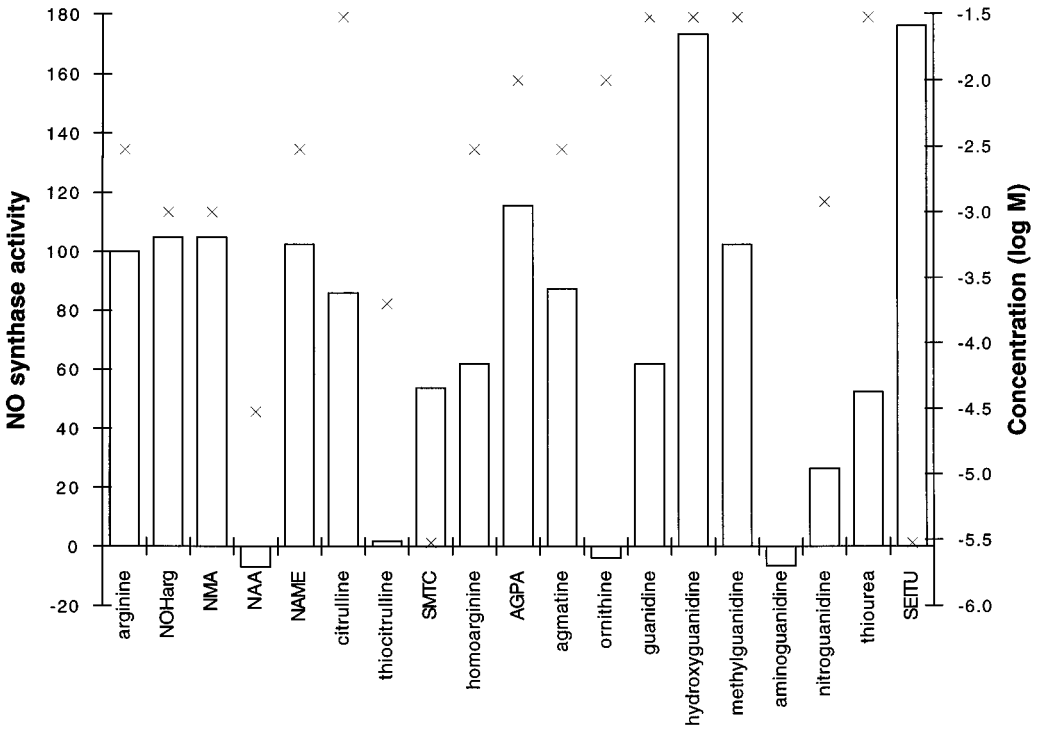


FIGURE 2: Analog-promoted dimerization of iNOS as measured by recovery of NO synthesis activity. An iNOS monomer preparation (0.4−0.6 μM) was incubated for 30 min in the presence of 4 μM H_4biopterin and a given substrate analog (concentrations are noted by ×), and then diluted 20-fold and assayed for NO synthesis activity as described in Materials and Methods. The rate of NO synthesis obtained from an incubation devoid of substrate or analog (set as zero in the figure) was substracted from each measurement and the corrected value was expressed as a percentage of the NO synthesis activity obtained with an incubation containing 3 mM L-arginine. The data come from a series of experiments in which the substrate- or analog-free NO synthesis activity ranged from 170 to 240 nmol/min per mg, and the activity in incubates containing L-arginine ranged from 470 to 710 nmol/min per mg.

1993). We thus evaluated the ability of analogs to promote the dimerization of iNOS. The iNOS monomer preparations used here had been formed by dissociating dimeric iNOS with urea, which typically results in an approximate 80:20 mixture of iNOS monomers and dimers (Abu-Soud *et al*., 1995). Because urea-generated monomers maintain most of their bound heme, they require only added H_4biopterin and L-arginine to dimerize (Abu-Soud *et al*., 1995).

Monomers were incubated for 30−90 min in the presence of 4 μM H_4biopterin and a chosen analog at a concentration that met or exceeded the concentration which gave maximal change in the NADPH oxidase activity of dimeric iNOS (see Figure 1). An aliquot of the incubation was then diluted and assayed for NO synthesis activity to estimate the amount of dimeric iNOS formed, since only dimeric iNOS can generate NO (Baek *et al*., 1993; Abu-Soud *et al*., 1995). The assay involved diluting the aliquot 20-fold in the presence of 3 mM L-arginine, which was usually enough to avoid residual competitive inhibition due to carryover of the analog. In some cases, gel filtration chromatography was also performed to confirm dimerization estimates that were obtained with the NO synthesis assay.

In the representative experiments shown in Figure 2, the degree of dimerization obtained in the presence of 4 μM H_4biopterin and 3 mM L-arginine was set as 100% in order to compare dimerization promoted by the analogs. Over several experiments, the recovered NO synthesis activity of incubates containing L-arginine and H_4biopterin ranged from 470 to 710 nmol of NO/min per mg ($n = 4$). On the basis of a specific activity for dimeric iNOS of 1200 nmol of NO/min per mg (Stuehr *et al*., 1991), this indicates that between 40% and 65% of the iNOS was present in dimeric form by the end of these incubations. In monomer incubates that did not receive L-arginine, recovered NO synthesis activity ranged from 50 to 250 nmol of NO/min per mg ($n = 4$), confirming that little or no dimerization occurs in the absence of L-arginine (Baek *et al*., 1993; Abu-Soud *et al*., 1995).

As shown in Figure 2, most of the L-arginine and guanidine analogs enabled the iNOS monomers to dimerize and recover their NO synthesis activity. As for the amino acids, NOHarg, NMA, NAME, AGPA, and citrulline promoted as much recovery as L-arginine, whereas SMTC, homoarginine, and agmatine promoted proportionally less. Ornithine, NAA, and thiocitrulline promoted no recovery of NO synthesis. Among the guanidine analogs, methylguanidine, hydroxyguanidine and SEITU all enabled reconstitution of iNOS NO synthesis activity. As shown, hydroxyguanidine and SEITU consistently proved better than L-arginine itself in promoting dimerization ($n = 3$). Guanidine, thiourea, and nitroguanidine were partly effective compared to L-arginine, and aminoguanidine was unable to reconstitute activity.

To independently assess dimerization, we utilized gel filtration chromatography to determine the proportion of dimer and monomer in the incubations. Figure 3 shows the gel filtration profiles of iNOS dimerization reactions that were run in the absence or presence of L-arginine or select analogs. In each chromatogram, the three peaks eluting at 7.5, 9, and 12 mL represent iNOS aggregates, dimer, and monomer, respectively. Inclusion of L-arginine or citrulline increased the dimer content of the incubates from and initial value of ∼20% to ∼45%, whereas inclusion of SEITU increased dimer assembly to a greater extent than L-arginine (∼60%), consistent with its promoting higher recovery of NO synthesis activity (Figure 2). Inclusion of aminoguani-

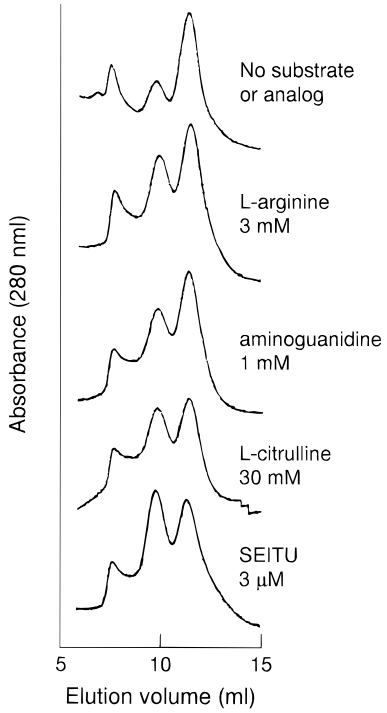


FIGURE 3: Gel filtration profiles for iNOS monomers incubated in the presence of 4 μM H_4biopterin plus no substrate, L-arginine, or the indicated analog. The incubations were carried out as in Figure 2. The protein peaks eluting at 7.5, 9, and 12 mL represent iNOS aggregates, dimer, and monomer, respectively. The data shown are representative of two or three separate experiments.

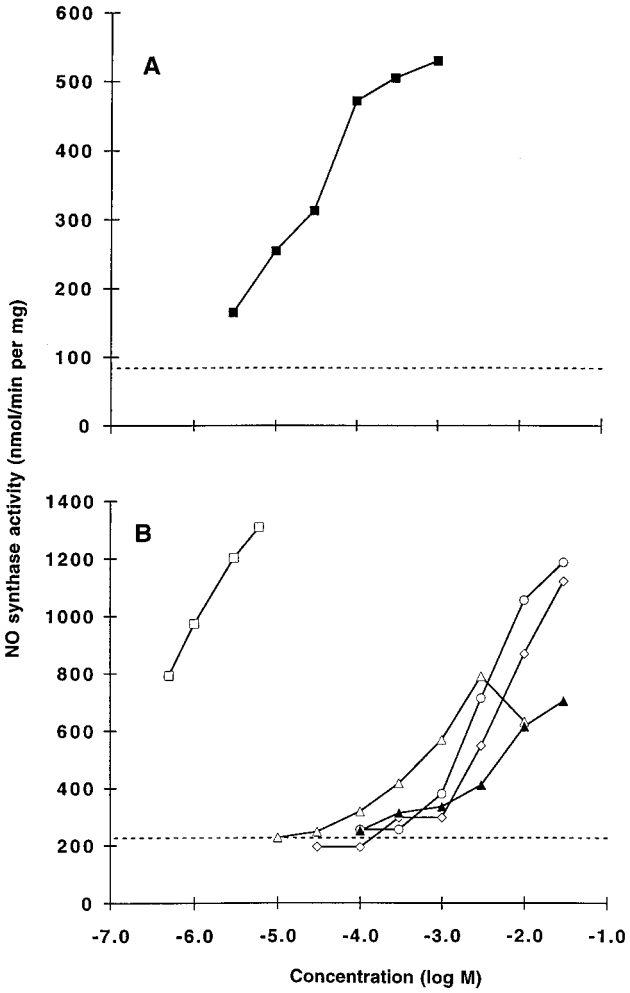


FIGURE 4: Dimerization of iNOS monomers as a function of L-arginine or analog concentration. Incubations containing iNOS monomers, H_4biopterin, and various concentrations of L-arginine (panel A) or analogs (panel B) were carried out as in Figure 2. The analogs used were (□) SEITU; (△) agmatine; (○) hydroxyguanidine; (◇) AGPA; and (▲) citrulline. After 30 min, the incubate was diluted and its NO synthesis activity determined in duplicate. The dashed line notes the activity obtained in incubates that did not contain L-arginine or an analog (negative control). The experiments shown are representative of two or three separate trials.

dine also resulted in some dimer formation, in apparent contrast with activity measurements that showed no activity was recovered. This discrepancy was solved when we found that carryover of residual aminoguanidine was still enough to suppress iNOS NO synthesis in the activity assay. Gel filtration results from incubates containing NAA or thiocitrulline showed that these compounds allowed for little or no dimerization (data not shown), consistent with their promoting no recovery of catalytic activity (Figure 2). Thus, the correlation between dimer formation and proportional recovery of NO synthesis activity held when using analogs in place of L-arginine.

We next determined the concentration response for L-arginine and five analogs regarding their ability to promote dimerization of iNOS monomers, as determined by recovery of NO synthesis activity. As shown in Figure 4, panel A, L-arginine displayed an EC_{50} of approximately 27 μM, and approached maximal dimerization at concentrations $\geq$300 μM. As shown in panel B, the five analogs promoted dimerization over a wide range of concentrations. SEITU was effective even when present at a concentration that approached that of the iNOS monomer, which was present at 0.4 μM. The four other analogs required near-millimolar concentrations to cause dimerization. Using the highest activity values obtained as an endpoint, the EC_{50}s for agmatine, hydroxyguanidine, citrulline, and AGPA were estimated to be 0.62, 2.9, 4.1, and 5.4 mM, respectively.

DISCUSSION

In this report, we evaluated the structural components that govern substrate interaction with iNOS by examining the ability of several L-arginine and guanidine analogs to bind to the enzyme, shift the spin state equilibrium, alter NADPH oxidase activity, support NO synthesis and promote subunit dimerization. To interpret the results, it is useful to note that L-arginine binds close enough to the NOS heme iron to influence both its spin state equilibrium (McMillan & Masters, 1993) and its binding interaction with small ligands such as NO and CO (Wang *et al*., 1993, 1994). Mechanistic considerations (Marletta, 1994; Griffith & Stuehr, 1995), as well as results obtained with substrate analogs (Garvey *et al*., 1994), suggest that the guanidinium of L-arginine is positioned closest to the heme iron, with the amino acid portion of the molecule binding at a distinct site some distance from the heme, as summarized in Figure 5 (adapted from Garvey *et al*., 1994). Figure 5 is referred to in discussing the various effects of the analogs.

Analog Structure and Binding Affinity. Because most of the analogs we tested did not support NO synthesis, binding affinities were estimated based on their changing the NADPH oxidase activity of dimeric iNOS. The estimated binding constants obtained by this method were slightly higher but in general agreement with published K_m or K_s values when available (i.e., for L-arginine, NOHarg, NMA, and NAME)

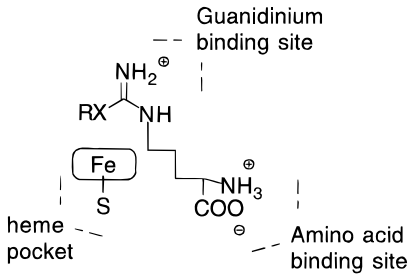


FIGURE 5: Model for analog binding within iNOS. X can be O, S, C, or N, whereas R can be aliphatic, aromatic, or heteroatom substituents. Adapted from Garvey *et al*. (1994).

(Stuehr *et al*., 1991; Olken & Marletta, 1993; Abu-Soud *et al*., 1994a; McMillan & Masters, 1993). Analog binding to iNOS was also indicated by a positive or negative shift in heme iron spin state equilibrium, as occurs with L-arginine (McMillan & Masters, 1993).

Regarding L-arginine, our results indicate that its α-aminopentanoic acid portion is not required for binding, in that guanidine itself can bind to iNOS. However, this side chain or one containing only an α-amino group (i.e., agmatine) increases the binding affinity up to 100-fold over guanidine alone. An amino acid side chain that is shortened or lengthened with respect to L-arginine (AGPA and homoarginine) still increases the binding affinity above guanidine, but to a lesser extent than L-arginine itself. In the absence of the guanidinium moiety (ornithine), the amino acid side chain appears to bind with very low affinity. Together, this suggests a role for the amino acid side chain, and in particular the positively-charged α-amino group, in properly positioning the guanidinium moiety for maximal interaction with its binding site.

The presence of the α-aminopentanoic acid side chain also increased the binding affinity of N-hydroxy-, N-nitro-, and N-methyl-substituted guanidines, albeit to a lesser degree than with guanidine alone. The exception was aminoguanidine, which had similar affinity as its amino acid homolog NAA. Why only the N-amino substitution confers increased affinity over N-hydroxy, N-methyl, or N-nitro substituents in the absence of the amino acid side chain is unclear. The spectroscopic data show that all four analogs promote a high spin shift, suggesting that none of them derive increased binding affinity via coordination of a heteroatom to the heme iron (Schenkman *et al*., 1967; Frey *et al*., 1994). It should be noted that unlike L-arginine analogs, N-substituted guanidines can conceivably bind in two ways, with the substituted nitrogen facing away from or toward the space normally occupied by the amino acid backbone (see Figure 5). Whether this occurs and affects the binding affinity of guanidine analogs is unknown.

Dimeric iNOS displayed poor binding affinity toward citrulline, consistent with citrulline being a terminal product of the reaction that requires rapid dissociation from the enzyme. This contrasts with thiocitrulline, which, on the basis of the spectral and kinetic data (Frey *et al*., 1994; Abu-Soud *et al*., 1994a), binds to iNOS with surprisingly high affinity. Both compounds shift the heme iron spin equilibrium toward low-spin upon binding. In the case of thiocitrulline, the shift toward low-spin may involve partial coordination of its sulfur to the NOS heme iron (Frey *et al*., 1994; Salerno *et al*., 1995). However, it is presently unknown how much sulfur coordination actually occurs or whether coordination can explain the increased affinity of thiocitrulline over citrulline. Indeed, thiourea, which displays a much weaker binding affinity than thiocitrulline, mimicks thiocitrulline both in causing a shift toward low-spin and in decreasing NADPH oxidation by iNOS. This suggests that thiourea and thiocitrulline may bind to iNOS in the same general manner within the guanidinium binding site, but additional factors such as the presence of the amino acid side chain and/or its positioning of the thiourea group may underlie the increased affinity toward thiocitrulline. An opposite result was obtained in the case of the isothiourea (SEITU), which has a slightly higher affinity toward iNOS than its related amino acid homolog SMITC. These both give a high-spin shift (Garvey *et al*., 1994; Furfine *et al*., 1994), suggesting that the isothiourea sulfur does not coordinate to the heme iron in either case. Apparently, neither heteroatom coordination nor an amino acid-containing side chain can explain the remarkable affinity of SEITU for the guanidinium binding site. In general, the high affinity iNOS displays toward analogs containing uncharged (thiocitrulline, SEITU, SMITC) or weakly basic functional groups (NOHarg and NAME) indicate that a positive charge is not required for tight binding within the guanidium binding site.

Analog Effect on iNOS NADPH Oxidation and NO Synthesis. In NOS, NADPH-derived electrons pass through the flavins to the heme iron, which is thought to bind and activate oxygen for mixed function oxidation of the substrate. In the absence of L-arginine, reduction of the iNOS heme iron still occurs (Abu-Soud & Stuehr, 1993), but leads to heme iron-catalyzed reduction of O_2 (Heinzel *et al*., 1992) and uncoupled NADPH oxidation. In the current study, L-arginine increased the NADPH oxidase activity of dimeric iNOS 6−8-fold over the substrate-free level, as reported previously (Abu-Soud *et al*., 1994a), indicating that it increases electron flux through iNOS in conjunction with initiating NO synthesis. The reaction intermediate NOHarg also increased NADPH oxidation in conjunction with NO synthesis, but exhibited a higher EC_{50} value and a smaller maximal increase in NADPH oxidation (3-fold) relative to L-arginine. Because NADPH oxidation in the presence of L-arginine or NOHarg is tightly coupled to NO synthesis, these differences are consistent with NOHarg having a slightly higher K_m and V_{max} than L-arginine with respect to NO synthesis, but requiring only one-third the amount of NADPH to generate NO (L-arginine requires 1.5 NADPH be oxidized per NO formed, whereas NOHarg requires only 0.5 NADPH per NO; Stuehr *et al*., 1991). Thus, iNOS NADPH oxidation increases in the presence of L-arginine or NOHarg to rates that are consistent with their different V_{max} values and NADPH stoichiometry requirements for NO synthesis. Homoarginine and NMA also increased iNOS NADPH oxidation, but in these cases NADPH oxidation was either slightly uncoupled (homoarginine; 2.5 mol of NADPH/mol of NO) or greatly uncoupled (NMA; ∼200 mol of NADPH/mol of NO; Olken & Marletta, 1993) with respect to NO synthesis. Uncoupling in the case of NMA may be due to its requiring N-demethylation prior to or following N-hydroxylation in order to generate NO (Olken & Marletta, 1993).

Several analogs were found to increase iNOS NADPH oxidation without supporting any detectable NO synthesis. This group includes guanidine, hydroxyguanidine, methylguanidine, and two compounds strikingly similar to L-

arginine (AGPA, agmatine). Conversely, six analogs decreased iNOS NADPH oxidation approximately 80% relative to the substrate free control. Although no guanidines supported NO synthesis, they each mimicked their amino acid homolog in increasing or decreasing iNOS NADPH oxidase activity. This indicates that the composition of the guanidine moiety is the primary determinant mediating the effect. Guanidine, hydroxyguanidine, and methylguanidine also increased iNOS NADPH oxidation to rates beyond those achieved with either L-arginine or the corresponding amino acid homologs. Although the reason for this is unknown, it is not due to their promoting dissociation of the iNOS dimer into monomers (data not shown), which have very poor NADPH oxidase activity (Abu-Soud *et al*., 1995). Together, we conclude that simple occupancy of the iNOS guanidinium binding site can up- or down-regulate electron flux through iNOS independent of NO synthesis. In contrast, the coupling of NADPH oxidation to NO synthesis is extremely structure sensitive and involves more than simple occupancy of this binding site. For example, coupling appears to require the presence of an amino acid side chain. The side chain may properly position the guanidine moiety relative to the heme iron for insertion of activated oxygen, or may enable the hydroxylated intermediate to remain bound and correctly positioned in iNOS so that it can undergo the second step in NO synthesis.

A variety of evidence indicates that the rate of NADPH oxidation by iNOS directly reflects the rate at which electrons are transferred through its heme iron (Baek *et al*., 1993; Abu-Soud & Stuehr, 1993; Abu-Soud *et al*., 1994a). Our current data have thus identified several L-arginine and guanidine analogs that can positively or negatively modulate electron flux through the iNOS heme iron, often independent of NO synthesis. In the cytochromes P450, substrate or substrate analog binding can stimulate heme iron reduction by increasing the heme iron reduction potential (Sligar & Murray, 1986). A similar situation appears to exist in neuronal NOS, in that heme iron reduction is increased upon binding L-arginine (Matsuoka *et al*., 1994) and decreased upon binding thiocitrulline or NAME (Abu-Soud *et al*., 1994a). On the basis of the available data, L-arginine or guanidine analogs that are substituted with electron-rich heteroatoms or groups (i.e., oxygen, sulfur, amino, or nitro groups) tend to down-regulate NADPH oxidation by iNOS. Some of these compounds (thioureas, ureas) also cause a low-spin shift in the iNOS heme iron spin state, which in cytochrome P450cam is associated with a decrease in heme iron reduction potential (Fisher & Sligar, 1985). However, the correlation for iNOS is not absolute because three analogs that decrease its NADPH oxidase activity (aminoguanidine, NAA, and NAME) also shift the spin equilibrium toward high-spin. In addition, these analogs do not appear to inhibit electron flux through the heme by coordinating directly to the iNOS heme iron. Thus, we speculate that binding of electron-rich analogs in the iNOS guanidinium binding site may decrease heme iron reduction through an electrostatic effect, and down-regulate iNOS NADPH oxidation as a result. This proposal is consistent with work done with myoglobin that showed point mutations which increase the electron density in the distal heme binding pocket lowered the heme iron reduction by 200 mV (Varadarajan *et al*., 1989).

Analog-Promoted Subunit Dimerization. Many of the L-arginine and guanidine analogs used in this study could substitute for L-arginine in promoting iNOS dimerization. The analog concentrations that promote half-maximal dimer assembly appear to be somewhat higher than the EC_{50} concentrations derived from NADPH oxidation measurements with dimeric iNOS. However, the analogs follow the same rank order in both cases, suggesting a common binding site for each affect. There was no apparent correlation between an analog's ability to promote dimerization and its acting as a substrate, shifting the heme iron spin equilibrium in a particular direction, or increasing versus decreasing iNOS NADPH oxidase activity. This argues that occupancy of the guanidinium binding site is the primary determinant for promoting dimerization, and occurs independent of specific electronic or catalytic changes involving the heme iron.

Of the 18 analogs tested, two consistently promoted dimerization better than L-arginine (hydroxyguanidine and SEITU). Curiously, the corresponding amino acid homologs (NOHarg and SMITC) did not promote excess dimerization, suggesting that the amino acid side chain may actually antagonize the effect. Two analogs (thiocitrulline and NAA) were judged ineffective at promoting iNOS dimerization, although they both bound tightly to the dimeric enzyme and decreased its NADPH oxidase activity. Whether thiocitrulline and NAA are ineffective at promoting dimerization because they cannot interact properly with monomeric iNOS will require further investigation. Again, the presence of the amino acid side chain appeared to exert a negative effect, in that thiourea and aminoguanidine both promoted some dimerization.

CONCLUSION

L-Arginine effects iNOS in at least four ways: it shifts the heme iron spin equilibrium toward high-spin, increases NADPH oxidation, serves as a substrate for NO synthesis, and promotes subunit dimerization. Of the four, NO synthesis is the most structurally specific, in that it requires that both a guanidinium and amino acid function be present in the substrate molecule. In contrast, a number of structural analogs can bind to the enzyme and mimick L-arginine's three other affects without supporting NO synthesis. In these cases, each analog appears to regulate its specific effect on the heme environment and reactivity or subunit dimerization through interacting with the guanidinium binding site of iNOS. Among the analogs that were inactive regarding NO synthesis were those which bind to iNOS with higher affinity, stimulate more electron flux, and promote more dimerization than L-arginine itself.

ACKNOWLEDGMENT

We thank Drs. Vernon Anderson, Denis Rousseau, Owen Griffith, Husam Abu-Soud, and Paul Feldman for helpful discussion, and Pam Clark for expert technical assistance.

REFERENCES

Abu-Soud, H. M., & Stuehr, D. J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 10769−10772.

Abu-Soud, H. M., Feldman, P. L., Clark, P., & Stuehr, D. J. (1994a) *J. Biol. Chem. 269*, 32318−32326.

Abu-Soud, H. M., Yoho, L. L., & Stuehr, D. J. (1994b) *J. Biol. Chem. 269*, 32047−32050.

Abu-Soud, H. M., Loftus, M., & Stuehr, D. J. (1995) *Biochemistry 34*, 11167−11175.

Baek, K. J., Thiel, B. A., Lucas, S., & Stuehr, D. J. (1993) *J. Biol. Chem. 268*, 21120−21129.
Boullerne, A. I., Petry, K. G., Meynard, M., & Geffard, M. (1995) *J. Neuroimmunol. 60*, 117−124.
Bredt, D. S., & Snyder, S. H. (1994) *Annu. Rev. Biochem. 63*, 175−195.
Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., & Snyder, S. H. (1991) *Nature 351*, 714−718.
Cho, H. J., Xie, Q.-w., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., & Nathan, C. (1992) *J. Exp. Med. 176*, 599−604.
Feelisch, M., & Noack, E. A. (1987) *Eur. J. Pharmacol. 139*, 19−30.
Feldman, P. L., Griffith, O. W., Hong, H., & Stuehr, D. J. (1993) *J. Med. Chem. 36*, 491−496.
Fisher, M. T., & Sligar, S. G. (1985) *J. Am. Chem. Soc. 107*, 5018−5019.
Frey, C., Narayanan, K., McMillan, K., Spack, L., Gross, S. S., Masters, B. S., & Griffith, O. W. (1994) *J. Biol. Chem. 269*, 26083−26091.
Furfine, E. S., Harmon, M. F., Paith, J. E., & Garvey, E. P. (1993) *Biochemistry 32*, 8512−8517.
Furfine, E. S., Harmon, M. F., Paith, J. E., Knowles, R. J., Salter, M., Kiff, R. J., Duffy, C., Hazelwood, R., Oplinger, J. A., & Garvey, E. P. (1994) *J. Biol. Chem. 269*, 26677−26683.
Garvey, E. P., Oplinger, J. A., Tanoury, G. J., Sherman, P. A., Fowler, M., Marshall, S., Harmon, M. F., Paith, J. E., & Furfine, E. S. (1994) *J. Biol. Chem. 269*, 26669−26676.
Ghosh, D. K., & Stuehr, D. J. (1995) *Biochemistry 34*, 801−807.
Griffith, O. W., & Stuehr, D. J. (1995) *Annu. Rev. Physiol. 57*, 707−736.
Heinzel, B., John, M., Klatt, P., Böhme, E., & Mayer, B. (1992) *Biochem. J. 281*, 627−630.
Klatt, P., Schmidt, K., Uray, G., & Mayer, B. (1993) *J. Biol. Chem. 268*, 14781−14787.
Klatt, P., Schmidt, K., Brunner, F., & Mayer, B. (1994) *J. Biol. Chem. 269*, 1674−1680.
Komori, Y., Wallace, G. C., & Fukuto, J. M. (1994) *Arch. Biochem. Biophys. 315*, 213−218.
Kroncke, K.-D., Fehsel, K., & Kolb-Bachofen, V. (1995) *Biol. Chem. Hoppe-Seyler 376*, 327−343.
Marletta, M. A. (1994) *Cell 78*, 927−930.
Matsuoka, A., Stuehr, D. J., Olson, J. S., Clark, P., & Ikeda-Saito, M. (1994) *J. Biol. Chem. 269*, 20335−20339.
McCartney-Francis, N., Allen, J. B., Mizel, D. E., Albina, J. E., Xie, Q.-w., Nathan, C. F., & Wahl, S. M. (1993) *J. Exp. Med. 178*, 749−754.
McMillan, K., & Masters, B. S. S. (1993) *Biochemistry 32*, 9875−9880.
McMillan, K., & Masters, B. S. S. (1995) *Biochemistry 34*, 3686−3693.
McMillan, K., Bredt, D. S., Hirsch, D. J., Snyder, S. H., Clark, J. E., & Masters, B. S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11141−11145.
Nakane, M., Klinghofer, V., Kuk, J. E., Donnelly, J. L., Budzik, G. P., Pollock, J. S., Basha, F., & Carter, G. W. (1995) *Mol. Pharmacol. 47*, 831−834.
Narayanan, K., & Griffith, O. W. (1993) *J. Med. Chem. 37*, 885−887.
Narayanan, K., Spack, L., McMillan, K., Kilbourn, R. G., Hayward, M. A., Masters, B. S. S., & Griffith, O. W. (1995) *J. Biol. Chem. 270*, 11103−11110.
Nathan, C., & Xie, Q.-w. (1994) *Cell 78*, 915−918.
Olken, N. M., & Marletta, M. A. (1993) *Biochemistry 32*, 9677−9685.
Ortiz de Montellano, P. R., Ed. (1986) *Cytochrome P-450: Structure, Mechanism, and Biochemistry*, Plenum Publishing Corp., New York.
Pou, S., Pou, N. S., Bredt, D. S., Snyder, S. H., & Rosen, G. M. (1992) *J. Biol. Chem. 267*, 24173−24176.
Pufahl, R. A., & Marletta, M. A. (1993) *Biochem. Biophys. Res. Commun. 193*, 963−970.
Pufahl, R. A., Nanjappan, P. G., Woodward, R. W., & Marletta, M. A. (1992) *Biochemistry 31*, 6822−6828.
Radomski, M. W., Palmer, R. M. J., & Moncada, S. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 10043−10047.
Salerno, J. C., Frey, C., McMillan, K., Williams, R. F., Masters, B. S. S., & Griffith, O. W. (1995) *J. Biol. Chem. 270*, 27423−27428.
Schenkman, J. B., Remmer, H., & Estabrook, R. W. (1967) *Mol. Pharmacol. 3*, 113−123.
Schmidt, H. H. H. W., & Walter, U. (1994) *Cell 78*, 919−925.
Sheta, E. A., McMillan, K., & Masters, B. S. S. (1994) *J. Biol. Chem. 269*, 15147−15153.
Sligar, S. G. & Murray, R. I. (1986) in *Cytochrome P-450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., Ed.) pp 429−503, Plenum Publishing Corp., New York.
Sono, M., Stuehr, D. J., Ikedasaito, M., & Dawson, J. H. (1995) *J. Biol. Chem. 270*, 19943−19948.
Stamler, J. S. (1994) *Cell 78*, 931−936.
Steuhr, D. J., & Ikeda-Saito, M. (1992) *J. Biol. Chem. 267*, 20547−20550.
Stuehr, D. J., Kwon, N. S., Nathan, C. F., Griffith, O. W., Feldman, P. L., & Wiseman, J. (1991) *J. Biol. Chem. 266*, 6259−6263.
Varadarajan, R., Zewert, T. E., Gray, H. B., & Boxer, S. G. (1989) *Science 243*, 69−72.
Wang, J., Stuehr, D. J., Ikeda-Saito, M., & Rousseau, D. L. (1993) *J. Biol. Chem. 268*, 22255−22258.
Wang, J., Rousseau, D. L., Abu-Soud, H. M., & Stuehr, D. J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 10512−10516.
Wang, J., Stuehr, D. J., & Rousseau, D. L. (1995) *Biochemistry 34*, 7080−7087.
White, K. A., & Marletta, M. A. (1992) *Biochemistry 31*, 6627−6631.
Wolff, D. J., & Gribin, B. J. (1994) *Arch. Biochem. Biophys. 311*, 293−299.
Wolff, D. J., & Lubeskie, A. (1995) *Arch. Biochem. Biophys. 316*, 290−301.
Wolff, D. J., Datto, G. A., Samatovicz, R. A., & Tempsick, R. A. (1993) *J. Biol. Chem. 268*, 9425−9429.
Yokoi, I., Kabuto, H., Habu, H., Inada, K., Toma, J., & Mori, A. (1994) *Neuropharmacology 33*, 1261−1265.

BI952844E