Substrate Specificity of NO Synthases: Detailed Comparison of L-Arginine, Homo-L-arginine, Their N^{ω}-Hydroxy Derivatives, and N^{ω}-Hydroxynor-L-arginine†

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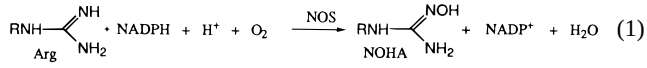
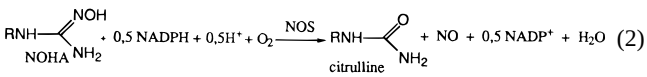
ABSTRACT: A detailed comparison of the oxidation of five compounds closely related to L-arginine (Arg) by purified recombinant neuronal and macrophage NO synthases (NOS I and NOS II) was performed. Homo-L-arginine (homo-Arg) is oxidized by both NOSs in the presence of NADPH with major formation of NO and homo-L-citrulline, with a molar ratio of close to 1, and minor formation of N^{ω}-hydroxyhomo-L-arginine (homo-NOHA). Oxidation of homo-NOHA by the two NOSs also leads to NO and homocitrulline in a 1:1 molar ratio. On the contrary, N^{ω}-hydroxynor-L-arginine (nor-NOHA) is a very poor substrate of NOS I and II, which fails to produce significant amounts of nitrite. The catalytic efficiency of both NOSs markedly decreases in the order Arg > NOHA > homo-Arg > homo-NOHA, as shown by the 20- and 10-fold decrease of k_{cat}/K_m observed for NOS I and NOS II, respectively, when comparing Arg to homo-NOHA. The greater loss of catalytic efficiency for homo-Arg, when compared to that for Arg, appears to occur at the first step (N-hydroxylation) of the reaction. In that regard, it is noteworthy that the V_m values for NOHA and homo-NOHA oxidation are very similar (about 1 and 2 μmol of NO min^{-1} mg of $protein^{-1}$ for NOS I and II, respectively). In fact, lengthening of the Arg chain by one CH_2 leads not only to markedly decreased k_{cat}/K_m but also to clear disturbances in NOS functioning. This is shown by a greater accumulation of the N^{ω}-hydroxyguanidine intermediate (homo-NOHA:homocitrulline ratio between 0.2 and 0.4) and an increased consumption of NADPH for NO formation (between 2.0 and 2.6 mol of NADPH consumed for the formation of 1 mol of NO in the case of homo-Arg, instead of 1.5 mol in the case of Arg). Most of the above results could be interpreted by comparing the possible positionings of the various substrates relative to the two NOS active oxygen species which are believed to be responsible for the two steps of the reaction.

Nitric oxide (NO) is a small gaseous molecule that serves as a mediator of many physiological events. NO is generated by a family of enzymes called NO synthases (NOSs)[1] which use L-arginine as a substrate. Three distinct isoforms of NOSs, which differ in their modes of regulation and functions, have been identified in mammalian tissues (for recent reviews, see for instance refs *1*−*3*). Neuronal and endothelial NOSs (NOS I and III) are constitutively expressed in an inactive form and require Ca^{2+}-dependent calmodulin (CaM) binding to activate their NO synthesis. In contrast, expression of macrophage NOS (NOS II) is only observed following induction by immunoactive cytokines such as interferon-γ or interleukin-2. Once expressed, macrophage NOS continuously generates NO in a Ca^{2+}-independent manner because it contains CaM as a tightly bound prosthetic group.

NOSs catalyze the two-step oxidation of a guanidino nitrogen of L-arginine to citrulline and NO, with formation of N^{ω}-hydroxy-L-arginine (NOHA) as an intermediate (eqs 1 and 2).



(1)



(2)

The two steps occur with consumption of NADPH and O_2; measurements performed on NOS I and II have indicated that 1.5 mol of NADPH is consumed to generate 1 mol of NO from 1 mol of arginine (*4*, *5*). More recent data under single-turnover conditions have unambiguously demonstrated that only one electron, i.e., 0.5 equiv of NADPH, is

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[1] Abbreviations: Arg, L-arginine; homo-Arg, homo-L-arginine; NOHA, N^{ω}-hydroxy-L-arginine; homo-NOHA, N^{ω}-hydroxyhomo-L-arginine; nor-NOHA, N^{ω}-hydroxynor-L-arginine; BH_4, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NADPH, nicotinamide adenine dinucleotide phosphate (reduced); DTT, D,L-dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; NDA, 2,3-naphthalenedicarboxaldehyde; PMSF, phenylmethanesulfonyl fluoride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NOS, NO synthase; CaM, calmodulin; SOD, superoxide dismutase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; GC, gas chromatography; HPLC, high-performance liquid chromatography.

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consumed to convert NOHA to citrulline and NO (*6*), the two other electrons being consumed to produce NOHA from arginine.

Several prosthetic groups (FAD, FMN, heme, and BH_4) are required to ensure proper activation of dioxygen and to give the active conformation to the enzyme. The catalytic site includes BH_4 and the heme group, an iron protoporphyrin IX, which is bound to the apoprotein by the thiolate moiety of a cysteine residue (*7*−*9*). NO synthases are homodimeric enzymes, and recent crystallographic data on a truncated NOS II suggest that the substrate guanidino group binds to the E371 carboxyl group, which should position the guanidine directly above the heme (*10*).

Even though the mechanism of NO formation is not fully established yet, much data on NOSs themselves and on closely related heme−thiolate proteins, the cytochromes P450, indicate that two iron species, a high-valent iron−oxo intermediate [presumably a porphyrin cation radical−Fe(IV)═O species] and the iron(II)−dioxygen [Fe(II)−O_2 or Fe(III)−$OO^{\bullet}$] complex of the catalytic cycle of dioxygen activation, are involved in the first and second step, respectively (*11*−*14*).

Up to now, very few compounds have been clearly shown to act as substrates for NOSs with oxidation of their guanidine or *N*-hydroxyguanidine function to the corresponding urea and NO. L-Arginine and NOHA are the endogenous substrates of NOSs with K_m values in the range of 1−10 μM for L-arginine (*4*, *8*, *9*, *15*−*19*) and 7−28 μM for NOHA (*4*, *18*, *20*−*22*) depending on the isoforms and their origins. Besides these two substrates, two other compounds were clearly reported to be capable of NOS-dependent generation of NO: N^{ω}-methyl-L-arginine, a widely used inhibitor of NOSs, which inactivates NOS I and NOS II (*20*, *23*−*25*) by causing heme loss, and homo-L-arginine (homo-Arg) (*26*−*31*). However, in the case of homo-Arg, the nature of the organic products was not fully determined. N^{ω}-Methoxy-L-arginine was also suspected of being a substrate for purified NOS I and NOS II on the basis of NADPH consumption (*32*) but was not studied in more detail.

Other arginine analogues such as canavanine (*31*) and ϵ-guanidinocaproic acid (*31*) were proposed to be NOS substrates as they generate NO in intact tissues or crude homogenates. However, these results have never been confirmed with purified NOSs, and the nature of the transformation of these compounds is not known. Similarly, agmatine and N^{ω}-hydroxyagmatine (*31*, *33*), as well as L-tyrosyl-L-arginine (*34*), were proposed to be precursors for NO synthesis as they caused endothelium-dependent vasorelaxation of rat aortic rings or accumulation of nitrite in LPS-treated glial cell cultures. Recent data suggest however that L-tyrosyl-L-arginine is first hydrolyzed by peptidases with release of arginine, which is most probably responsible for NO production (*35*).

The very limited number of substrates of NOSs known so far suggests that highly specific structural features are required for action by these enzymes. In an effort to better understand these structural requirements and to determine the relationship between the substrate structure and the enzyme response, we have synthesized a series of compounds bearing a guanidine, amidine, *N*-hydroxyguanidine, or amidoxime function, which are more or less closely related to L-arginine. This paper reports a detailed comparative study of the oxidation by NOS I and II of the closest analogues of L-arginine (Arg), namely, homo-L-arginine (homo-Arg) and three N^{ω}-hydroxy derivatives: NOHA, N^{ω}-hydroxyhomo-L-arginine (homo-NOHA), and N^{ω}-hydroxynor-L-arginine (nor-NOHA). It shows that homo-Arg and homo-NOHA are oxidized by NOS I and II with formation of homo-L-citrulline and NO, whereas nor-NOHA is a very poor substrate of those NOSs. Moreover, even the small increase of the L-arginine chain length by one CH_2 leads to a marked decrease of the NOS catalytic efficiency, particularly for the N-hydroxylation step, and to a partial uncoupling of NADPH consumption from NO formation. These results may be interpreted by considering possible positionings of these substrates relative to the NOS active oxygen species.

MATERIALS AND METHODS

Materials

(6*R*)-5,6,7,8-Tetrahydro-L-biopterin (BH_4) was purchased from Alexis (Coger, Paris, France). NADPH, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase (from yeast) were from Boehringer Mannheim Biochemicals. L-Arginine, homo-L-arginine, L-citrulline, bovine brain calmodulin, bovine erythrocytes superoxide dismutase (SOD), bovine liver catalase, bovine hemoglobin, bovine serum albumin, *Aspergillus* nitrate reductase, CaM−Sepharose, and 2′,5′-ADP−agarose resins were purchased from Sigma. Sephadex G50 medium and Ni−nitrilotriacetate T Sepharose CL4B resins were products of Pharmacia. Other reagents for colorimetric assays, HPLC, and syntheses were purchased from Aldrich, Fluka, or Janssen unless otherwise indicated.

Syntheses

NOHA and homo-NOHA were prepared by a modification of the procedure of Wallace and Fukuto (*36*) with N^{δ}-(benzyloxycarbonyl)-L-ornithine and N^{ϵ}-(benzyloxycarbonyl)-L-lysine (from Bachem) as starting materials (*37*, *38*). Synthesis of nor-NOHA was performed in seven steps from L-glutamine (from Acros) as described recently (*38*).

Homo-L-citrulline is a side product of homo-NOHA synthesis at the penultimate step which involves protected N^{ϵ}-cyanolysine and hydroxylamine. The hydrochloride salt of homo-L-citrulline was obtained after stirring overnight in HCl/dioxane as previously described for related compounds (*36*). Treatment of the precipitate with 0.8 N ammonia in water yielded the free base which was lyophilized to afford a white solid: $R_f = 0.25$ (5:5:0.5:1 $CHCl_3$/MeOH/H_2O/CH_3CO_2H); ^{13}C NMR (D_2O, 250 MHz) δ 24.5, 31.6, 32.9, 42.4, 57.6, 164.5, 177.7 ppm; MS (chemical ionization, NH_3) *m*/*z* (relative intensity) 190 (25%), 172 (50%), 147 (100%).

Protein Expression and Purification

NOS I. Recombinant NOS I expression was achieved according to a published method (*39*) using a slightly modified procedure. The previously described NOS I-V60 plasmid (*40*) was used to transform the W(RΔ) yeast strain following a lithium acetate protocol (*41*). W(RΔ) derives from strain W303-1.B (*MAT*α; *ade-2*-1; *his-3*; *leu-2*; *ura-3*; *trp-1*) by disruption of the cytochrome P450 reductase 1 gene with a TRP1 selection marker (*42*, *43*). This strain was

chosen since it was found that boosted NOS expression resulted from the lack of cytochrome P450 reductase. Recombinant yeasts were cultivated in a shaking incubator at 28 °C using 2 L flasks filled with 200 mL of glucose-based yeast minimum medium (SGI) (per liter, 7 g of yeast nitrogen base, 1 g of Bacto casamino acids, 5 g of glucose, and 30 mg of L-tryptophan) for 24 h up to a cell density of 3×10^7 cells/mL. Two hundred milliliters of yeast extract (YE) medium (per liter, 20 g of yeast extract, 20 g of Bactopeptone, and 4% v/v ethanol) was added to the culture for another 24 h until the cell density reached $1-2 \times 10^8$ cells/mL. Galactose (final concentration of 3% v/v) was then added at the late log phase, and cells were allowed to incubate at 28 °C for 12 h prior to being harvested at 4 °C. The cell pellet was resuspended in the minimum amount of buffer A [50 mM Tris-HCl (pH 7.4), 1 mM Arg, 150 mM NaCl, 3 mM DTT, 5 μM BH_4, 2 mM $CaCl_2$, 20% glycerol, 1 mM PMSF, 10 μg/mL pepstatin, 10 μg/mL leupeptin, and 10 μg/mL aprotinin], and cells were disrupted by using glass beads and vigorous shaking. The crude extract was centrifuged twice at 4 °C for 10 min at 10000*g*, and 4 mL of calmodulin−Sepharose resin was stirred in the supernatant for 30 min, after extra addition of PMSF. NOS I was batch purified upon centrifugation of the resin (3000*g* for 5 min) with repetitive washes (same buffer) until no more protein could be detected in the supernatant. Bound NOS I was then eluted from the resin upon washing with small amounts of buffer A in which $CaCl_2$ was replaced by 5 mM EGTA. An equal volume of approximately 30% glycerol was added, and the protein was quickly frozen in liquid nitrogen before being stored at −80 °C. Typically, this procedure yielded 22 nmol of NOS I/L (3.2 L total volume) which displayed absorption at 450 nm after dithionite reduction and CO bubbling. NOS I was estimated to be more than 90% pure by SDS−PAGE. To remove residual EGTA, the protein was quickly purified on a Sephadex G50 column prior to being used.

NOS II. Recombinant NOS II was isolated and purified from *Escherichia coli* as described previously (*19*). *E. coli* was transformed with a plasmid containing mouse NOS II with a six-histidine tag on its N terminus and a plasmid that contained human calmodulin. Purification was achieved through binding of the crude extract to nickel affinity resin, washing the resin to remove unbound proteins, and eluting with imidazole. The eluted protein was concentrated and subjected to 2′,5′-ADP affinity chromatography. NOS II was estimated to be more than 90% pure by SDS−PAGE (*19*).

Protein Determination

Protein concentrations were determined by the method of Bradford (*44*) using bovine serum albumin as a standard and the Bradford reagent from Bio-Rad.

Hemoglobin Assay

The initial rate of NO synthesis was determined at 37 °C using the classical spectrophotometric oxyhemoglobin assay for NO (*45*, *46*). Briefly, 10−20 μL aliquots containing NOS, 5 μM BH_4, and 2−5 mM DTT were added to a prewarmed cuvette that contained 50 mM HEPES (pH 7.4), supplemented with 15−20 μM oxyhemoglobin, 100 units/mL SOD, 100 units/mL catalase, 200 μM NADPH, 4 μM FAD, 4 μM FMN, 5 μM BH_4, and substrate at the desired concentration, to give a final volume of 150 μL. In the case of NOS I, 1 mM $CaCl_2$ and 10 μg/mL CaM were also present. The reference cuvette had the same composition except that 50 mM HEPES, 5 μM BH_4, and 2−5 mM DTT were added instead of NOS-containing solutions. The NO-mediated conversion of oxyhemoglobin to methemoglobin was monitored over time as an increase in absorbance at 401 nm and quantitated using an extinction coefficient of 38 mM^{-1} cm^{-1} (*45*).

Kinetic Constant Determinations

K_m and V_m were determined by plotting the initial rate of NO formation versus substrate concentration. Curves were fitted to $y = (V_m S)/(K_m + S)$ using the software Kaleidagraph, version 3.0.2.

NADPH Consumption

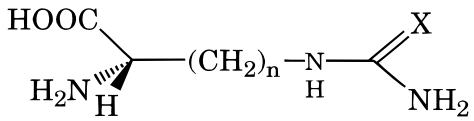
NADPH consumption was followed by monitoring the decrease in absorbance at 340 nm and quantitated using an extinction coefficient of 6.2 mM^{-1} cm^{-1} under conditions identical to those used in the hemoglobin assay, except that no oxyhemoglobin was present to avoid interference.

Measurement of NO_2^- and NO_3^- Formation

Determinations were performed according to a previously described procedure (*47*). Incubated mixtures containing 50 mM HEPES (pH 7.4), 100 μM NADPH, 4 μM FAD, 4 μM FMN, 6 μM BH_4, 0.4−1 mM DTT, and 0.5−1 μg of NOS in a 100 μL final volume were shaken at 37 °C for the indicated time. For NOS I, 1 mM $CaCl_2$ and 10 μg/mL CaM were also added. Reactions were quenched with 25 μL of ethanol and the mixtures allowed to stay at room temperature for at least 1 h before addition of the Griess reagent [125 μL of 1% sulfanilamide in 0.5 N HCl and 125 μL of 0.1% *N*-(1-naphthyl)ethylenediamine in 0.5 N HCl] (*47*). Absorbances were measured at 543 nm. Calibration curves were made from identical incubated mixtures without NOS and containing various concentrations of $NaNO_2$ to properly determine the amounts of NO_2^- formed in the enzymatic reactions. When nitrate determination was necessary, reactions were stopped by a 3 min incubation in a boiling water bath and reduction of nitrate to nitrite was performed on a 30 μL aliquot of the incubated mixture, following a previously described protocol (*48*). Twenty-four microliters of a solution containing a 5:5:5:1 mixture of 5 mM glucose 6-phosphate, 600 units/L nitrate reductase, 100 μM NADPH, and 100 units/mL glucose-6-phosphate dehydrogenase was added, and the mixture was incubated at 28 °C for 30 min. Calibration curves were made from identical incubated mixtures without NOS but containing various KNO_3 concentrations to check the completion of nitrate reduction. The sum of NO_2^- and NO_3^- was determined after addition of 2 volumes (108 μL) of Griess reagent.

Identification and Quantitation of Metabolites from NOS-Dependent Oxidation of Homo-Arg

Study by GC−MS. Amino acids present in incubated mixtures of NOS with homo-Arg and NADPH were derivatized after lyophilization of the samples according to a procedure described by Meyer et al. (*49*). The dried residue



X = NH **n = 4 : homo-Arg**
n = 3 : Arg

X = N-OH **n = 4 : homo-NOHA**
n = 3 : NOHA
n = 2 : nor-NOHA

X = O **n = 4 : homocitrulline**
n = 3 : citrulline

FIGURE 1: Structure of the compounds used in this study.

was first heated at 80 °C for 1 h in the presence of 2 N HCl in methanol. After evaporation under a stream of nitrogen, samples were treated with pentafluoropropionic acid anhydride at 80 °C for 1 h and evaporated again. The resulting pentafluoropropionyl methyl ester derivatives were dissolved in methylene chloride prior to GC analysis (Hewlett-Packard 5890 gas chromatograph with a HP-5 fused silica capillary column that was 30 m × 0.32 mm, 0.25 μm film thickness, and with helium as the carrier gas). The temperature increase was as follows: 2 min at 140 °C, linear increase (6 °C/min) up to 240 °C, and 2 min at 240 °C. Coinjection of standard solutions permitted identification of the peaks. GC−MS spectra (chemical ionization + NH_3 with a Riber Mag R10-10 C mass spectrometer) of standard solutions of citrulline and homocitrulline showed molecular peaks at *m*/*z* of 481 and 495, respectively.

Study by HPLC. Amino acid products were derivatized and separated by reverse-phase HPLC according to the procedure recently described by Clague et al. (*50*). Thus, 50 μL of a sample containing 20 μM phenylalanine was mixed in HPLC vials with 100 μL of 0.1 M potassium borate (pH 9.5), 10 μL of 0.5 M NaCN in the same borate buffer, and 50 μL of 10 mM 2,3-naphthalenedicarboxaldehyde (NDA) in methanol and the mixture allowed to stay at room temperature for 15 min. Samples were applied to a Nova-Pak C_{18} column (150 mm × 3.9 mm, 4 μm particle size, Waters Associates Inc.), which was maintained at 40 °C and equilibrated with 60% 5 mM ammonium acetate buffer (pH 6.0) (solvent A) and 40% methanol (solvent B). Elution conditions were as follows: 2 min at 40% solvent B, a linear increase from 40 to 60% solvent B over 20 min and from 60 to 100% over the next 5 min, followed by 3 min of 100% methanol, and a return to the initial conditions over the last 3 min. The flow rate was 0.5 mL/min, and detection was performed at 420 nm. Phenylalanine was used as an internal standard. Retention times were as follows: Arg, 10.7 min; NOHA, 10.3 min; citrulline, 5.3 min; homo-Arg, 13.3 min; homo-NOHA, 12.7 min; homocitrulline, 6.7 min; and phenylalanine, 19.9 min.

RESULTS

(1) Nitrite Formation upon NOS-Dependent Oxidation of L*-Arginine Analogues by NADPH and O_2.* The three *N*-hydroxyguanidines, homo-NOHA (*38*), NOHA (*36*, *37*), and nor-NOHA (*38*) (Figure 1), were synthesized as described recently. Their activity as substrates of NOS, as well as that of Arg and homo-Arg, was first tested by measuring

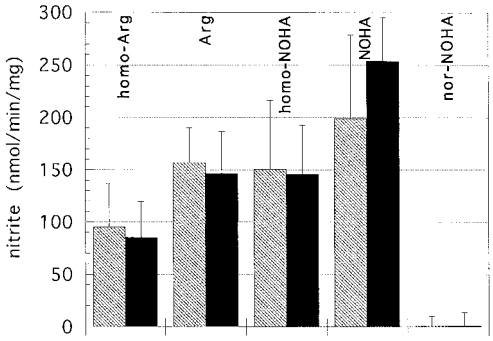


FIGURE 2: Nitrite formation upon oxidation of L-arginine analogues by purified recombinant NOS I and II (hatched and black bars, respectively). Incubations were carried out for 10 min as indicated in Materials and Methods using 100 μM substrate and 0.5−1 μg of NOS in 100 μL of 50 mM HEPES (pH 7.4) buffer containing 4 μM FAD, 4 μM FMN, 6 μM BH_4, 20 units/mL superoxide dismutase, 0.4−1 mM DTT, and 100 μM NADPH. Amounts of NO_2^- (mean values ± SD from three to five experiments) are expressed in nanomoles per minute per milligram of protein.

their ability to produce nitrite in the presence of NADPH and O_2. Two NOSs were studied, recombinant NOS II from murine macrophages, which was expressed in *E. coli* (*19*), and mouse neuronal NOS I which was expressed in yeast (*39*, *40*).

As expected, Arg and NOHA led to high amounts of NO_2^- with both NOSs (Figure 2). Their longer homologues, homo-Arg and homo-NOHA, also appeared to be good substrates for NOS I and II, with levels of NO_2^- formation only moderatly decreased when compared to those for Arg and NOHA, respectively. However, quite interestingly, nor-NOHA appeared to be a very poor substrate for NOS I and II, as it did not lead to significant amounts of NO_2^- using the Griess method (*47*) (Figure 2). Similar results were obtained when higher concentrations of nor-NOHA (up to 500 μM) were used. Thus, the following kinetic experiments have only been performed on the four other NOS substrates.

(2) Characterization of the NOS-Dependent Oxidations of Homo-Arg and Homo-NOHA. Formation of NO_2^- from homo-Arg absolutely requires the presence of both NOS, either I or II, and NADPH. It is a typical NOS-dependent reaction as it is almost completely inhibited in the presence of 1 mM N^{ω}-nitro-L-arginine, a classical inhibitor of NOSs (*1*−*3*) (data not shown).

Moreover, as expected, NO_2^- formation from homo-Arg is completely suppressed in the absence of calmodulin in the case of NOS I. Very similar results were obtained with homo-NOHA, as well as with Arg and NOHA (data not shown).

Two techniques were used to determine the nature of the organic products that were formed besides nitrogen oxides during the NOS-dependent oxidation of homo-Arg and homo-NOHA. The first one was gas chromatography (GC) of the reaction mixture after derivatization of the amino acid functions (*49*) [methylation of the COOH function and amide formation for the NH_2 functions with $(CF_3CF_2CO)_2O$; see Materials and Methods]. A new peak with a retention time identical to that of derivatized, synthetic homocitrulline was detected after NOS-dependent oxidation of homo-Arg and homo-NOHA. Coupling of GC with mass spectrometry gave a mass spectrum of the metabolite identical to that of

Table 1: Products Obtained after NOS-Dependent Oxidation of Arg, NOHA, Homo-Arg, and Homo-NOHA[a]

		urea[b]	*N*-OH-Gua[c]	$[NO_2^- + NO_3^-]$	urea[b]:$[NO_2^- + NO_3^-]$	*N*-OH-Gua:urea[b]
NOS I	Arg	420 ± 59	0 ± 0	555 ± 87	0.8	0
	NOHA	434 ± 75	−	444 ± 120	1.0	−
	homo-Arg	342 ± 88	80 ± 10	287 ± 51	1.2	0.2
	homo-NOHA	583 ± 110	−	519 ± 98	1.1	−
NOS II	Arg	349 ± 58	60 ± 10	323 ± 22	1.1	0.2
	NOHA	372 ± 82	−	456 ± 75	0.8	−
	homo-Arg	274 ± 31	106 ± 15	236 ± 47	1.2	0.4
	homo-NOHA	276 ± 38	−	215 ± 59	1.3	−

[a] NO_2^- and NO_3^- formations were measured by the colorimetric assays described in Materials and Methods. Quantitation of the N^{ω}-hydroxyguanidine products and of the ureido products was carried out after HPLC separation and by using authentic samples as standards. Results are expressed as nanomoles of product per minute per milligram of protein (mean values ± SD from at least four experiments). Incubations were run for 10 min as indicated in Materials and Methods using 100 μM substrate (except 200 μM for homo-Arg and homo-NOHA and NOS I) and 0.5−2.0 μg of NOS in 200 μL of 50 mM HEPES buffer (pH 7.4) containing 6 μM BH_4, 4 μM FAD, 4 μM FMN, and 300 μM NADPH. [b] The word urea is used here to indicate a compound containing a urea function; it does not correspond to NH_2CONH_2 itself but to citrulline or homocitrulline which are N-substituted ureas. [c] *N*-OH-Gua = NOHA or homo-NOHA.

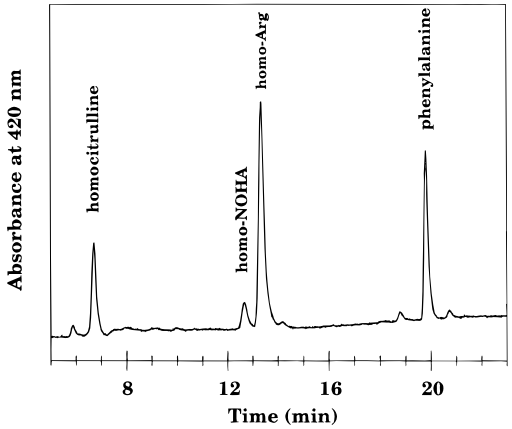


FIGURE 3: HPLC chromatogram of an incubated mixture of homo-Arg with NOS II (after derivatization with 2,3-naphthalenedicarboxaldehyde). Incubation conditions were as in Figure 2 except that the NADPH concentration was 300 μM. HPLC analysis conditions were as described in Materials and Methods.

authentic derivatized homocitrulline (with a molecular ion at $m/z = 495$) (data not shown).

The second technique used was HPLC after derivatization of the amino acid functions by a method recently described for NOHA and N^{δ}-cyanoornithine (*50*). This technique also showed that oxidation of homo-NOHA either by NOS I or by NOS II gave only one new product with a retention time identical to that of authentic homocitrulline (after derivatization). However, oxidation of homo-Arg by NOS I led to two new products with retention times identical to those of homo-NOHA and homocitrulline (in a 0.2:1 ratio under the conditions used) (Table 1). Interestingly, oxidation of Arg by NOS I under similar conditions was found to only lead to citrulline by the same method. Oxidation of homo-Arg by NOS II also generated homo-NOHA and homocitrulline in a markedly higher ratio (0.4, Table 1 and Figure 3). Under identical conditions, oxidation of Arg by NOS II produced citrulline as a major product, but also small amounts of NOHA (about 15%, Table 1).

(3) Stoichiometry of the NOS-Dependent Oxidations of Arg and Homo-Arg. Simultaneous detection of NO_2^- and NO_3^-, the final stable oxidation products of NO under aerobic conditions, and of citrulline (by the HPLC method mentioned above), after oxidation of Arg and NOHA by either NOS I or NOS II, gave a urea:$[NO_2^- + NO_3^-]$ molar ratio of ∼1 (Table 1), consistent with previous literature data on NOSs of various origins (*51*−*53*) (eqs 1 and 2). With homo-Arg and homo-NOHA as substrates, the urea (homocitrulline): $[NO_2^- + NO_3^-]$ ratios obtained with NOS I and NOS II were also very close to 1, although slightly higher than that found for Arg and NOHA (Table 1).

Table 2: Stoichiometry of NADPH Consumption for NO Formation in NOS-Dependent Oxidation of Arg, Homo-Arg, NOHA, and Homo-NOHA[a]

	Arg	NOHA	homo-Arg	homo-NOHA
NOS I	1.5 ± 0.7[b]	1.0 ± 0.5[c]	2.6 ± 0.7[d]	1.1 ± 0.5[e]
NOS II	1.6 ± 0.3[f]	0.4 ± 0.05[g]	2.0 ± 0.1[h]	0.5 ± 0.05[i]

[a] Results are expressed in moles of NADPH consumed per mole of NO formed (mean value ± SD from three to six experiments). Experiments were carried out under the conditions described in Figure 2 except for the NADPH concentration (200 μM) and the presence of SOD and catalase (100 units/mL). Two almost identical incubations were performed. In the first one (not containing hemoglobin), NADPH consumption was followed at 340 nm. In the second one, oxyhemoglobin was added and NO formation was monitored by the hemoglobin assay (*45*). [b] With 50 μM Arg. [c] With 125 μM NOHA. [d] With 200 μM homo-Arg. [e] With 700 μM homo-NOHA. [f] With 125 μM Arg. [g] With 500 μM NOHA. [h] With 300 μM homo-Arg. [i] With 1.2 mM homo-NOHA.

We then measured the amounts of NADPH required for the formation of 1 mol of NO during NOS-dependent oxidation of Arg, homo-Arg, and their N^{ω}-hydroxy derivatives. Nitric oxide production was monitored by the classical hemoglobin assay (*45*, *46*). Table 2 shows that the NOS II-dependent formation of NO from Arg and NOHA required the consumption of 1.6 ± 0.2 and 0.4 ± 0.1 mol of NADPH. These values are very close to the theoretical values corresponding to the stoichiometry of eqs 1 and 2. They are consistent with those previously published for purified NOS II under steady-state conditions (*4*). They also are consistent with very recent data showing that NOS I, under single-turnover conditions, consumes only one electron (0.5 equiv of NADPH) in oxidizing NOHA to citrulline and NO (*6*).

In the case of NOS II-dependent oxidation of homo-NOHA, a value very similar to that observed for NOHA (0.5 ± 0.05) was obtained, suggesting a very similar behavior of NOS II toward the oxidation of these *N*-hydroxyguanidines. However, during NOS II-dependent oxidation of homo-Arg, 2.0 ± 0.1 mol of NADPH was required to produce 1 mol of NO (Table 2). In fact, Table 1 shows that, during formation of 1 mol of homocitrulline, 0.4 mol of homo-NOHA is also

Table 3: Kinetic Parameters for NOS-Dependent Oxidation of Arg, Homo-Arg, and Their N^{ω}-Hydroxy Derivatives at 37 °C[a]

	NOS I			NOS II		
	K_m[b]	V_m[c]	k_{cat}/K_m[d]	K_m[b]	V_m[c]	k_{cat}/K_m[d]
Arg	2.7 ± 0.5	0.8 ± 0.1	1.58	13 ± 2	1.5 ± 0.6	0.50
NOHA	13 ± 2	1.2 ± 0.5	0.48	59 ± 2	2.0 ± 0.3	0.14
homo-Arg	23 ± 5	0.5 ± 0.1	0.11	33 ± 8	0.8 ± 0.2	0.11
homo-NOHA	68 ± 15	1.0 ± 0.2	0.08	146 ± 21	1.7 ± 0.4	0.05

[a] K_m and V_m were determined for NO formation by NOS I and NOS II under conditions described in Materials and Methods (similar to those described in Figure 2 and Table 2, by using the hemoglobin assay). Values are expressed as means ± SD of at least four separate experiments.[b] In μM. [c] In μmol min^{-1} mg of protein^{-1}. [d] In μM^{-1} s^{-1} (calculated by using molecular masses of 320 and 260 kDa for NOS I and NOS II, respectively).

produced. Thus, after consumption of 2.0 ± 0.1 mol of NADPH, 1 mol of NO, about 1 mol of homocitrulline, and 0.4 mol of homo-NOHA are formed. Assuming that 0.4 mol of NADPH is required for the formation of 0.4 mol of homo-NOHA from monooxygenation of homo-Arg, it appears that 1.6 ± 0.1 mol of NADPH is consumed for the formation of NO and homocitrulline. This value is very similar to that expected for oxidation of homo-Arg into homocitrulline and NO in two steps as in eqs 1 and 2.

In the case of NOS I-dependent oxidations of Arg and NOHA, values of NADPH consumption similar to those found in the case of NOS II were observed, despite the relatively great standard deviations measured for this recombinant NOS. However, the mean value found for Arg oxidation was close to the theoretical value of 1.5 mol of NADPH consumed per mole of NO formed (Table 2). Interestingly enough, oxidation of homo-Arg to NO by NOS I requires significantly more NADPH than oxidation of Arg (2.6 ± 0.7 mol instead of 1.5 ± 0.7). Part of these 2.6 ± 0.7 mol of NADPH required for formation of 1 mol of NO and 1 mol of homocitrulline is consumed in the concomitant production of homo-NOHA (0.2 mol according to Table 1). However, it seems that NOS I consumes more NADPH to produce NO from homo-Arg (2.4 ± 0.7) than from Arg (1.5 ± 0.7).

(4) Kinetics of NOS I and II-Dependent Oxidation of Arg, Homo-Arg, and Their N^{ω}-Hydroxy Derivatives. K_m and V_m values for NOS-dependent oxidation of the four substrates to NO at 37 °C are reported in Table 3, along with the deduced k_{cat}/K_m values that are indicative of the catalytic efficacy of the NOSs for these substrates. For both NOSs, the best k_{cat}/K_m values were obtained for Arg (1.58 and 0.5 μM^{-1} s^{-1} for NOS I and NOS II, respectively). Then, k_{cat}/K_m decreases in the order Arg > NOHA > homo-Arg > homo-NOHA. In fact, NOS I and NOS II are 15- and 5-fold more efficient toward Arg than toward homo-Arg, respectively. They are also 6- and 3-fold more efficient for NOHA than for homo-NOHA, respectively. Interestingly enough, the V_m values of NOS I- and NOS II-dependent oxidation of NOHA and homo-NOHA are very similar (between 1 and 2 μmol of NO min^{-1} mg of protein^{-1}). They correspond to high activities (between 260 and 640 turnovers min^{-1}). Thus, the different catalytic efficiencies of NOSs for NOHA and its higher homologue are mainly due to an increase of K_m (Table 3). For both NOSs, guanidines are better substrates than their N^{ω}-hydroxy derivatives, mainly because of markedly lower K_m values (by a factor between 3 and 5), since the V_m values are slightly higher for N^{ω}-hydroxyguanidines.

DISCUSSION

The aforementioned results show that homo-Arg and its N^{ω}-hydroxy derivative, homo-NOHA, act as good substrates of NOS I and II, in a manner similar to that of Arg and NOHA. They establish for the first time that NOS I- and NOS II-dependent oxidation of homo-Arg leads not only to homocitrulline and NO, in a ratio of ~1, but also to homo-NOHA. Oxidation of homo-NOHA then leads to homocitrulline and NO in a 1:1 ratio. It is thus very likely that the major reaction pathway occurring with homo-Arg (eqs 3 and 4) is very similar to that demonstrated in the case of Arg (eqs 1 and 2).

$$\text{homo-Arg} \xrightarrow[\text{NOS}]{\text{NADPH} + \text{O}_2} \text{homo-NOHA} \quad (3)$$

$$\text{homo-NOHA} \xrightarrow[\text{NOS}]{\text{NADPH} + \text{O}_2} \text{homocitrulline} + \text{NO} \quad (4)$$

Interestingly, the shorter analogue of NOHA, nor-NOHA, is a very poor substrate of NOS I and II, as it leads to insignificant amounts of nitrite (at the level of the detection limit of the assay), even at nor-NOHA concentrations up to 500 μM.

NOS I seems to be specifically adapted to its natural substrate, Arg, as an increase of the Arg chain length by only one CH_2 group leads to a marked decrease of k_{cat}/K_m (Table 3). The loss of efficacy of NOS II when replacing Arg by homo-Arg (5-fold decrease of k_{cat}/K_m) also exists but to a lesser extent. This could be related to the greater ability of the NOS II active site to accommodate longer effectors, which has already been mentioned in the literature (*54*, *55*). The loss of efficacy of NOSs for the oxidation of homo-NOHA, when compared to NOHA, is smaller (6- and 3-fold decrease of k_{cat}/K_m for NOS I and II, respectively; Table 3). Moreover, the V_m values found for NOHA and homo-NOHA oxidation by NOS I and II are not significantly different. These results could be explained by considering the nature of the NOS substrate binding site and the mechanisms proposed for the two steps of arginine oxidation. Arginine binding to NOS seems to involve at least three sites of interaction which recognize the amino acid, the NH^{δ}, and the positively charged guanidinium functions (*2*) (Figure 4). The importance of the amino acid moiety in positioning arginine analogues has been revealed by studies on compounds lacking either the carboxylic or the amine function (*56*) and by comparison of L- and D-enantiomers (*56*). The major role played by the NH moiety linking the guanidine moiety to the side chain (NH^{δ} in Arg and NOHA) in substrate positioning was shown by the very low affinity of L-2-amino-

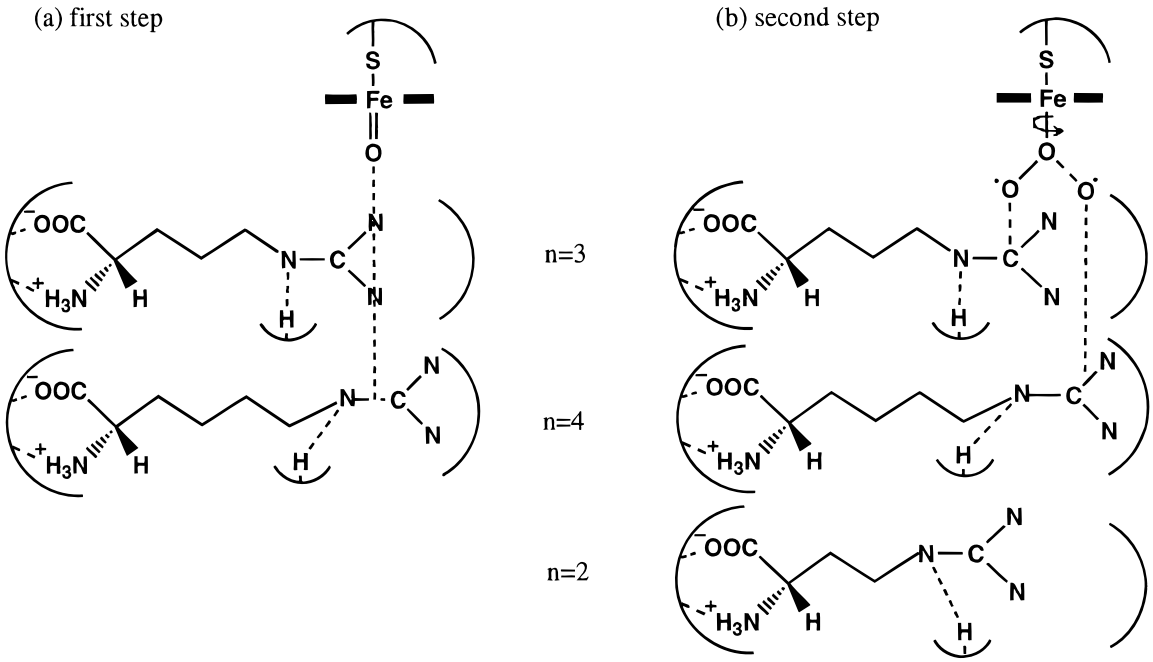


FIGURE 4: Hypothetical positionings of arginine analogues with respect to the active oxygen species possibly involved in the NOS catalytic cycle. This model is based on the assumption that the active species is an Fe(IV)═O porphyrin cation radical species for the first step (a) and Fe(III)−OO• for the second step (b). Substrates in panel a are Arg ($n = 3$) and homo-Arg ($n = 4$); they are NOHA ($n = 3$), homo-NOHA ($n = 4$), and nor-NOHA ($n = 2$) in panel b.

6-amidinohexanoic acid (indospicine) (*57*) and its N^{ω}-hydroxy derivative for NOSs (*37*). Crystallographic data indicate that aminoguanidine binding in the active site of a truncated NOS II not containing BH_4 depends on glutamate 371 and tryptophan 366, with glutamate 371 being a good candidate for interaction of NOS with the NH^{δ} of arginine (*10*).

Obviously, interactions with one or more of these binding sites will determine if a substrate is well-positioned to be processed by NOSs and at which rate this will occur. Our results suggest that, once positioned, homo-NOHA is oxidized by NOS I and II at the same rate as NOHA, whereas oxidation of homo-Arg is markedly slower than that of Arg. A possible, simple explanation of these results refers to the different active species involved in the first and second steps of NOS-dependent oxidation of Arg.

The terminal NH group of Arg should be well positioned relative to the high-valent iron−oxo species involved in the first step (*11*−*14*) to undergo an oxygen atom transfer. The terminal NH group of homo-Arg should not be as well positioned relative to the rigid Fe═O species because of its longer chain, and this could explain its slower oxidation. On the contrary, oxidation of NOHA and homo-NOHA, by addition of the Fe(II)−O_2 [Fe(III)−OO•] terminal oxygen atom to the *N*-hydroxyguanidine carbon (*11*−*14*), could occur at similar rates because of possible rotation of the Fe−OO• species around its Fe−O axis (Figure 4).

This model also explains why nor-NOHA is such a bad substrate of NOSs; this substrate is too short to position its central *N*-hydroxyguanidine carbon close to the terminal oxygen atom of the Fe−OO• species whatever its conformation (Figure 4).

The ideal positioning of Arg in the NOS active site results in an almost "perfect" stoichiometry of 1.5 mol of NADPH consumed for the formation of 1 mol of citrulline and 1 mol of NO. The stoichiometry calculated for NADPH consumption during homo-Arg oxidation catalyzed by NOS II (1.6 mol of NADPH for 1 mol of NO and about 1 mol of homocitrulline) (see the end of paragraph 3 in Results) is very similar to that found for Arg. However, the larger value obtained for NOS I-dependent oxidation of homo-Arg (2.3) would suggest a partial uncoupling of NADPH consumption from substrate oxidation. It could be due to an oxidase activity of NOS (*1*) consuming NADPH and O_2 to generate $O_2^{\bullet -}$, H_2O_2, or H_2O, instead of its oxygenase reaction leading to NO and homocitrulline, because of a less perfect positioning of homo-Arg in the active site. Another disturbance of the normal function of NOS when replacing Arg by homo-Arg is the accumulation of the N^{ω}-hydroxyguanidine intermediate during oxidation of homo-Arg by NOS I and NOS II, which is more important than during oxidation of Arg (Table 1). In fact, NOHA is either not detected (NOS I) or formed as a very minor product (NOS II) during oxidation of Arg, while homo-NOHA is always formed in significant amounts (20−40% compared to homocitrulline) during oxidation of homo-Arg. This should also be related to the less perfect positioning of homo-Arg in the active site. In that respect, it is noteworthy that there is a qualitative relationship between the amount of N^{ω}-hydroxy intermediate observed during the various reactions and the decrease of the corresponding k_{cat}/K_m values, with the order NOS II and homo-Arg > NOS I and homo-Arg ≥ NOS II and Arg > NOS I and Arg.

In conclusion, homo-Arg and homo-NOHA are the first reported compounds different from Arg and NOHA which act as good substrates of NOSs with a rate of formation of NO and the corresponding urea comparable to that obtained with L-arginine itself. However, even a slight change in the structure of Arg, such as lengthening of its chain by one CH_2, leads not only to a markedly decreased k_{cat}/K_m but also to clear disturbances in NOS functioning such as partial uncoupling of electron consumption from oxygen atom

transfer and the accumulation of N^{ω}-hydroxy intermediates. Finally, the strong substrate specificity of NOS I and II is illustrated by their very low activity toward nor-NOHA, which has only one fewer CH_2 than NOHA.

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