Oxidations of N^{ω}-Hydroxyarginine Analogues and Various *N*-Hydroxyguanidines by NO Synthase II: Key Role of Tetrahydrobiopterin in the Reaction Mechanism and Substrate Selectivity

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Oxidations of L-arginine **2**, homo-L-arginine **1**, their N^{ω}-hydroxy derivatives **4** and **3** (NOHA and homo-NOHA, respectively), and four *N*-hydroxyguanidines, N^{ω}-hydroxynor-L-arginine **5** (nor-NOHA), N^{ω}-hydroxydinor-L-arginine **6** (dinor-NOHA), *N*-(4-chlorophenyl)-*N*′-hydroxyguanidine (**8**), and *N*-hydroxyguanidine (**7**) itself, by either NOS II or (6*R*)-5,6,7,8-tetrahydro-L-biopterin (BH_4)-free NOS II, have been studied in a comparative manner. Recombinant BH_4-free NOS II catalyzes the oxidation of all *N*-hydroxyguanidines by NADPH and O_2, with formation of NO_2^- and NO_3^- at rates between 20 and 80 nmol min^{-1} (mg of protein)$^{-1}$. In the case of compound **8**, formation of the corresponding urea and cyanamide was also detected besides that of NO_2^- and NO_3^-. These BH_4-free NOS II-dependent reactions are inhibited by modulators of electron transfer in NOS such as thiocitrulline (TC) or imidazole (ImH), but not by Arg, and are completely suppressed by superoxide dismutase (SOD). They exhibit characteristics very similar to those previously reported for microsomal cytochrome P450-catalyzed oxidation of *N*-hydroxyguanidines. Both P450 and BH_4-free NOS II reactions appear to be mainly performed by $O_2^{\bullet-}$ derived from the oxidase function of those heme proteins. In the presence of increasing concentrations of BH_4, these nonselective oxidations progressively disappear while a much more selective monooxygenation takes place only with the *N*-hydroxyguanidines that are recognized well by NOS II, NOHA, homo-NOHA, and **8**. These monooxygenations are much more chemoselective (**8** being selectively transformed into the corresponding urea and NO) and are inhibited by Arg but not by SOD, as expected for reactions performed by the NOS $Fe^{II}-O_2$ species. Altogether, these results provide a further clear illustration of the key role of BH_4 in regulating the monooxygenase/oxidase ratio in NOS. They also suggest a possible implication of NOSs in the oxidative metabolism of certain classes of xenobiotics such as *N*-hydroxyguanidines, not only via their monooxygenase function but also via their oxidase function.

Introduction

In the past decade, nitric oxide (NO) has emerged as a major biological mediator in mammals (*1*). It is produced from L-arginine (Arg)[1] by heme thiolate proteins called NO synthases (NOSs). Three types of NOS have been characterized in mammals, based on amino acid sequence, tissue distribution, and expressional control (*2*). Two isoforms (soluble NOS I and membrane-bound NOS III) are constitutively expressed, and their activity depends on intracellular Ca^{2+} concentration which can trigger calmodulin binding and activation of NOS. The last isoform (NOS II) is regulated at the transcriptional level by cytokines and other infectious agents and is mostly independent of Ca^{2+} concentration since it contains calmodulin as a tightly bound prosthetic group. In the presence of NADPH and dioxygen, all three NOSs catalyze the two-step oxidation of Arg into nitric oxide and citrulline (Cit) as follows (*3*):

$$\text{Arg} + \text{NADPH} + \text{H}^+ + \text{O}_2 \xrightarrow{\text{NOS}} \text{NOHA} + \text{NADP}^+ + \text{H}_2\text{O}$$

$$\text{NOHA} + 0.5\text{NADPH} + 0.5\text{H}^+ + \text{O}_2 \xrightarrow{\text{NOS}} \text{Cit} + \text{NO} + 0.5\text{NADPH}^+ + \text{H}_2\text{O}$$

In the first step, Arg is N^{ω}-hydroxylated with insertion of one oxygen atom from dioxygen and consumption of

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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; dinor-NOHA, N^{ω}-hydroxydinor-L-arginine; OH-gua, *N*-hydroxyguanidine; Cit, L-citrulline; NMA, N^{ω}-methyl-L-arginine; TC, L-thiocitrulline; SEITU, *S*-ethylisothiourea; ImH, imidazole; BH_4, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; DTT, D,L-dithiothreitol; NDA, 2,3-naphthalenedicarboxaldehyde; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NOS, NO synthase; P450, cytochrome P450; SOD, superoxide dismutase; NO_2-Arg, N^{ω}-nitro-L-arginine.

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two electrons from NADPH to produce the stable intermediate N^{ω}-hydroxy-L-arginine (NOHA) (*4*). The second step which leads to the formation of nitric oxide and Cit is also a monooxygenation step, but only one electron from NADPH is necessary (*5*). The site for NO production is heme, but other cofactors are required: flavin adenine dinucleotide and flavin mononucleotide which serve in the transfer and storage of electrons derived from NADPH and tetrahydrobiopterin (BH_4) which seems to be involved at least in the allosteric modulation of NOS (*6*, *7*). Even though the presence of the heme thiolate is reminiscent of other monooxygenase enzymes such as cytochromes P450 (P450), NO synthases are unique among this family of enzymes in several ways. (1) NO formation proceeds with consumption of an odd number of electrons. Whereas the first step seems to involve the iron−oxo species "Fe^{V}=O" as is the case in most P450-dependent monooxygenations, the catalysis of the second step is generally attributed to the intermediate complexes ($Fe^{III}-OO^{\bullet}$ and $Fe^{III}-OO^{-}$) (*8*−*11*). (2) NO synthases are the only heme proteins involving BH_4 as a supplementary cofactor. Beyond its well-characterized effect on NOS quaternary structure, stability, and active site structure (*12*), BH_4 has also been proposed to control the redox properties of the heme (*13*), to influence the reactivity of the $Fe^{II}-O_2$ species (*14*), and even to deliver electrons to the heme (*15*, *16*). (3) NOSs are multimeric enzymes consisting of two identical subunits. Each subunit has a two-domain structure which allows the association of a reductase component responsible for electron transfer between NADPH and flavins and of an oxygenase component containing the binding sites for substrate, heme, and BH_4 which are all located in close proximity (*16*, *17*). Calmodulin binds at the junction between the two domains and controls electron transfer between the flavins and the heme (*1*−*3*). Thus, NO synthases are the most complex heme thiolate proteins known so far, and mechanistic studies are still needed to explain all the aspects of NO biosynthesis.

At present, NOSs are known to catalyze two different kinds of reactions. The first ones are monooxygenations that appear only to occur on few substrates. Arg, NOHA, and their homologues with one more CH_2 carbon, homo-L-arginine (homo-Arg) and N^{ω}-hydroxyhomo-L-arginine (homo-NOHA), are the only compounds oxidized by NOS with formation of NO and the corresponding urea with a very high catalytic efficiency (*18*). All the other tested Arg or NOHA derivatives either failed to produce NO, as described in the case of nor-Arg or nor-NOHA (*18*, *19*), or led to small amounts of NO, as in the case of N^{ω}-methyl-L-arginine (NMA) (*19*, *20*). However, quite recently, some *N*-aryl-*N*′-hydroxyguanidines have been reported to be oxidized by NOS II in a manner similar to that seen with NOHA, with V_m values for NO formation that are only 4 times lower than those found for NOHA (*21*).

The second kind of reaction catalyzed by NOSs is the reduction of O_2 into $O_2^{\bullet-}$ and H_2O_2. When NOS isoforms are saturated with cofactors and Arg, NADPH consumption is fully coupled to NO production, 1.5 equiv of NADPH being consumed per NO molecule produced (*4*, *5*). However, in the absence of Arg or when the enzyme is not fully loaded with BH_4, electrons coming from NADPH serve in oxygen reduction and lead to reduced oxygen species such as superoxide anion (*22*, *23*) and hydrogen peroxide (*24*, *25*). Superoxide generation has also been demonstrated in vivo and is suspected to be involved in several pathological conditions, alone or in conjunction with peroxynitrite which is the product of its reaction with nitric oxide (*26*−*28*). Thus, the oxidase activity of NOS may turn NO synthase into a superoxide/peroxynitrite synthase (*29*) and induce irreversible tissue damage.

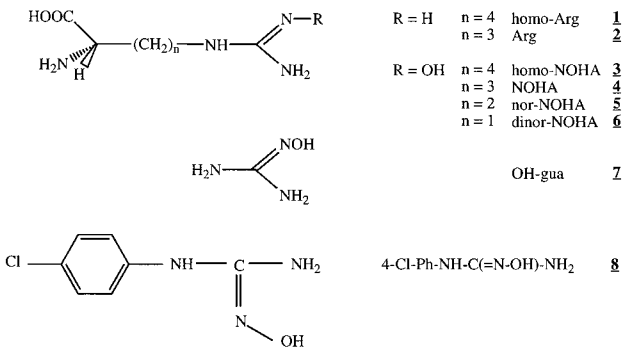


Figure 1. Structure of the compounds used in this study.

Interestingly, it has been recently reported that recombinant NOS not containing BH_4 was able to catalyze the oxidation of NOHA with formation of Cit, N^{δ}-cyano-L-ornithine, and NO_2^{-} (*30*).

To better understand the substrate specificity of NOSs, the effects of BH_4 on the substrate specificity and reaction mechanism of NOSs, we have compared the abilities of recombinant NOS II and BH_4-free NOS II to catalyze the oxidation of a series of *N*-hydroxyguanidines, including NOHA **4** and its analogues **3**, **5**, and **6**, *N*-(4-chlorophenyl)-*N*′-hydroxyguanidine **8**, and *N*-hydroxyguanidine ***7*** itself (Figure 1). All these compounds are oxidized with cleavage of their C=NOH bond, however, with very different rates and mechanisms when either NOS II or BH_4-free NOS II is used.

Materials and Methods

Materials. (6*R*)-5,6,7,8-Tetrahydro-L-biopterin and L-thiocitrulline were purchased from Alexis (Coger, Paris, France). NADPH came from Boehringer Mannheim Biochemicals. Arg, homo-Arg, *N*-hydroxyguanidine, Cit, N^{ω}-nitro-L-arginine, bovine erythrocyte superoxide dismutase (SOD), *Aspergillus* nitrate reductase, and bovine serum albumin were purchased from Sigma. Homo-NOHA, NOHA, nor-NOHA, and dinor-NOHA were synthesized following previously described procedures (*31*, *32*). *N*-(4-Chlorophenyl)-*N*′-hydroxyguanidine **8** was prepared in 65% yield by reacting cyanamide **8b** with hydroxylamine hydrochloride in anhydrous ethanol according to the procedure of Schantl and Türk (*33*). (4-Chlorophenyl)urea **8a** and 4-chlorophenylcyanamide **8b** were obtained in 90 and 75% yield by reacting 4-chloroaniline with potassium cyanate in 0.5 M HCl and cyanogen bromide in methanol, respectively, according to the method of Schantl and Türk (*33*). The compounds used in this study exhibited ^{1}H NMR and mass spectra in complete agreement with those reported previously (*31*−*33*). Other reagents were purchased from Aldrich.

Recombinant NOS II was isolated and purified from *Escherichia coli* in the absence of BH_4 as described previously (*34*).

Protein Quantitation. Protein concentrations were determined by the method of Bradford (*35*) using bovine serum albumin as a standard and the Bradford reagent from Bio-Rad.

Assessment of NO_2^{-} and NO_3^{-} Formation. Nitrite detection was performed according to a previously described procedure (*36*). Incubations containing 50 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.4), 500 μM substrate, 100 μM NADPH, 100 μM D/L-dithiothreitol (DTT), and 1 μg of NOS II in a final volume of 100 μL were shaken at 37

°C for 10 min, the reactions 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) (*36*). Absorbances were measured at 550 nm in a 96-well microplate, and standard curves with $NaNO_2$ in the presence of 100 μM NADPH were used to determine the amount of nitrite in the incubations. When nitrate quantification was necessary, incubations were stopped by heating for 3 min at 95 °C, and reduction of nitrate to nitrite was performed in the presence of nitrate reductase and an NADPH-regenerating system, following a previously described protocol (*18*, *36*).

NADPH Consumption. NADPH consumption was followed at 37 °C by monitoring the decrease in absorbance at 340 nm and quantitated using an extinction coefficient of 6.2 mM^{-1} cm^{-1}. Unless otherwise indicated, cuvettes containing 100 μM NADPH, 100 μM DTT, and 500 μM substrate in 150 μL of HEPES buffer (50 mM, pH 7.4) were allowed to stay at 37 °C for 2 min prior to the addition of 1.5 μg of NOS II to the sample cuvette.

HPLC Identification and Quantitation of Metabolites from NOS II-Dependent Oxidation of NOHA and 8. Amino acid products were derivatized with 2,3-naphthalenedicarboxaldehyde (NDA) in the presence of NaCN and separated by reverse-phase HPLC according to the procedure recently described by Clague et al. (*37*).

Incubations containing 500 μM *N*-(4-chlorophenyl)-*N*′-hydroxyguanidine **8**, 1 mM NADPH, and 2.5 μg of NOS II in 50 μL (final volume) of 50 mM HEPES (pH 7.4) were shaken at 37 °C for 5 min. The reactions were quenched with 50 μL of a 1:1 mixture of acetonitrile and 5 mM H_3PO_4 containing 40 μM 4-chlorobenzamide as an internal standard. Separation of the metabolites was performed as previously described (*38*) on a 250 mm × 4.6 mm Nucleosil ODS 5 μm column (SFCC-Shandon). The mobile phase was a gradient between solvent A [water containing 5 mM phosphoric acid (pH 2.6)] and solvent B (acetonitrile) using the following program: at 0 min, 10% B; at 5 min, linear gradient to 40% B over the course of 15 min; at 20 min, linear gradient to 100% B over the course of 10 min; at 30 min, linear gradient to 10% B over the course of 5 min followed by reequilibration for 15 min. The flow rate was 1 mL/min, and the absorbance was monitored at 240 nm (λ_{max} for **8**, **8a**, and **8b** are 232, 241, and 236 nm, respectively). The retention times for **8**, **8a**, **8b**, and 4-chlorobenzamide were 6.7, 19.5, 24.7, and 18.1 min, respectively. Calibration curves were made from identical mixtures containing various concentrations of **8**, **8a**, and **8b** but without NOS II.

Results

Oxidation of Compounds 1−8 with BH_4-Free NOS II. Whereas guanidines (homo-Arg and Arg) could not evoke any significant nitrite and nitrate production in the presence of BH_4-free NOS II and NADPH under our conditions (see Materials and Methods), all six *N*-hydroxyguanidines **3**−**8** produced variable amounts of nitrite and nitrate ions (Table 1). NOHA seems to be the most efficient substrate in the series, while nitrite and nitrate production from the unsubstituted *N*-hydroxyguanidine **7** was hardly detectable. Intermediate amounts of nitrite and nitrate were obtained with NOHA analogues **3**, **5**, and **6**, and with *N*-aryl-*N*′-hydroxyguanidine **8**. However, it is noteworthy that experiments using the hemoglobin assay for NO formation (*39*) showed that not one of compounds **1**−**8** was able to produce detectable amounts of NO in the presence of BH_4-free NOS II and NADPH (data not shown).

To gain further insight into the characteristics of the BH_4-free NOS II-dependent oxidation of *N*-hydroxyguanidines **3**−**8**, we have studied the influence of some classical effectors of NOSs on NO_2^- formation from NOHA itself and from compound **8**.

Table 1. Nitrite and Nitrate Production and NADPH Consumption by BH_4-Free NOS II in the Presence of Compounds 1−8

	activity [nmol min^{-1} (mg of protein)$^{-1}$][a]		
	nitrite production	nitrate production	NADPH oxidation
no substrate	≤1	≤5	45 ± 8
10 μM BH_4	≤1	nd[b]	112 ± 11
homo-Arg **1**	≤1	≤5	nd[b]
Arg **2**	≤1	≤5	nd[b]
homo-NOHA **3**	28 ± 10	22 ± 10	55 ± 7
NOHA **4**	49 ± 9	31 ± 10	92 ± 8
nor-NOHA **5**	26 ± 11	12 ± 4	50 ± 11
dinor-NOHA **6**	33 ± 15	13 ± 8	47 ± 7
OH-gua **7**	7 ± 5	10 ± 5	55 ± 2
4-Cl-Ph-NH-C(=N-OH)-NH_2 **8**	30 ± 6	18 ± 10	150 ± 30

[a] Nitrite and nitrate production were quantitated from incubations containing 100 μM NADPH, 100 μM DTT, 1 μg of BH_4-free NOS II, and 500 μM potential substrate (when present) in a total volume of 100 μL. Nitrite and nitrate formation was linear over time for at least 10 min. After 10 min at 37 °C, incubations were stopped by heating for 3 min at 90 °C and submitted to the Griess assay as described in Materials and Methods. The rate of NADPH oxidation was measured spectrophotometrically at 340 nm with reference and sample cuvettes having the same composition (100 μM NADPH and 100 μM DTT) except that BH_4-free NOS II (1.5 μg) was only present in the sample cuvette. Results are means (±SD) of four separate experiments. [b] Not determined.

(1) Characteristics of the Oxidation of NOHA with BH_4-Free NOS II. In the absence of NOHA, nitrite production was indistinguishable from background noise, showing that the observed nitrite production was directly linked to NOHA oxidation (Figure 2A).

Oxidation of NOHA was fully NOS- and NADPH-dependent since removal of either the enzyme or the cosubstrate led to a 90% decrease in the level of nitrite production (Figure 2A). Moreover, the level of NOHA oxidation increased linearly with BH_4-free NOS II concentration up to 0.1 mg/mL and with time up to 10 min at 37 °C (data not shown). More surprisingly, Arg, which is not a substrate under the conditions studied here but should compete with NOHA for binding in the active site, did not exhibit any inhibitory effect on the reaction (Figure 2A). A similar result was obtained with N^{ω}-methyl-L-arginine (NMA), a classical inhibitor of NOS, which even led to a slight increase in activity. Other NOS inhibitors such as L-thiocitrulline (TC), *S*-ethylisothiourea (SEITU), and imidazole (ImH), which act as substrate competitors but also as inhibitors of the electron transfer between flavins and heme (*40*), inhibit nitrite production by 60−70% when used at 1 mM. Thus, whereas electron transfer appears to be important for BH_4-free NOS II-dependent oxidation of NOHA, binding of NOHA in the active site does not seem to be required.

Finally, addition of very small amounts of SOD (10 units/mL) led to an almost complete inhibition of nitrite production (Figure 2A). This result is the most striking one since, in the presence of saturating amounts of BH_4, SOD failed to inhibit nitrite production from NOHA and NOS II at concentrations up to 100 units/mL (Figure 3A). Figure 3A also demonstrates that the concentration of SOD which allows the half-maximum effect in BH_4-free NOS II-catalyzed oxidation of NOHA is very low (0.6 unit/mL). Such spectacular inhibitory effects of SOD were

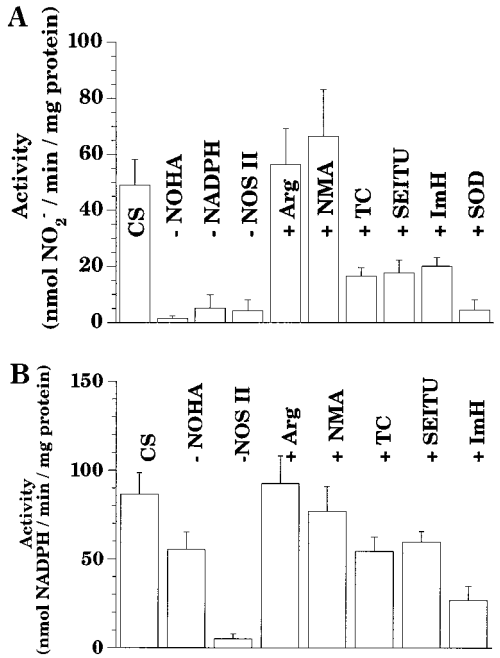


Figure 2. Influence of cofactors, inhibitors, and SOD on nitrite production (A) and NADPH oxidation (B) in oxidation of NOHA by BH_4-free NOS II. The control incubation (complete system, CS) was obtained with 500 μM NOHA under the conditions described in Table 1. In experiments regarding NADPH consumption, 100 μM NADPH was used. When mentioned, NOHA, NADPH, or NOS was removed and Arg, NMA, TC, SEITU, or ImH was added at a final concentration of 1 mM. The SOD concentration was 10 units/mL. Means (±SD) of four separate experiments.

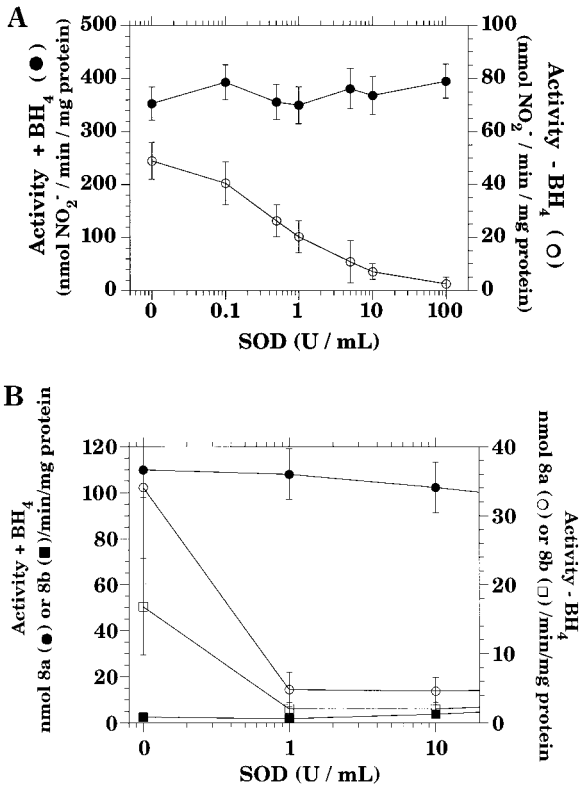


Figure 3. Effects of increasing amounts of SOD on nitrite production from NOHA and NOS II (A) and on the oxidation of **8** by NOS II (B). Incubations in the absence of BH_4 (white symbols) were carried out as described in Table 1 except that increasing concentrations of SOD were added. For the incubations in the presence of BH_4 (black symbols), 10 μM BH_4 and 120 μM DTT were also added. Activities in panel A are expressed in nanomoles of NO_2^- per minute per milligram of protein; in panel B, they are expressed in nanomoles of **8a** (circles) or **8b** (squares) per minute per milligram of protein [means (±SD) of four experiments]. Ordinate values indicated on the left are for BH_4-containing NOS II, whereas those on the right are for BH_4-free NOS II.

previously observed in the oxidation of several compounds containing a C=N−OH function, including *N*-hydroxyguanidines, with microsomal cytochromes P450 (*38*). In those reactions, small amounts of SOD (1−10 units/mL) were also sufficient to lead to almost complete inhibition of P450-dependent formation of NO_2^-.

Consequently, we suggest that superoxide ion may also be directly involved in NOHA oxidation by BH_4-free NOS II. Figure 2B shows the effects of the various compounds used in Figure 2A on NADPH consumption by BH_4-free NOS II in the presence of NOHA. As previously reported (*39*), the level of NADPH consumption is decreased in the absence of NOHA. Addition of Arg and NMA had weak effects on both NADPH consumption (Figure 2B) and NO_2^- formation (Figure 2A), whereas imidazole had a clear inhibitory effect on both NADPH consumption and NO_2^- formation. This parallel variation of electron consumption and NO_2^- formation in SOD-inhibited oxidation of NOHA by BH_4-free NOS II is in agreement with the involvement of $O_2^{\bullet-}$ in this oxidation. Other NOS inhibitors, TC and SEITU, had intermediate effects (Figure 2); they inhibited NO_2^- formation slightly more than NADPH consumption. This could be due to direct chemical reactions of these compounds with $O_2^{\bullet-}$, in competition with $O_2^{\bullet-}$-dependent oxidation of NOHA.

(2) NADPH Consumption during BH_4-Free NOS II-Dependent Oxidation of *N*-Hydroxyguanidines 3−8. Basal NADPH oxidation by BH_4-free NOS II in the absence of either the cofactor or substrate was found to occur at a relatively low rate of 45 ± 8 nmol min^{-1} (mg of protein)$^{-1}$ at 37 °C (Table 1), in agreement with the value obtained by Presta et al. under similar conditions (*39*). This rate was only slightly modified when one of the *N*-hydroxyguanidines (**3**, **5**, **6**, or **7**) was added (Table 1). These compounds do not seem to affect electron transfer from NADPH when used at concentrations as high as 500 μM. On the contrary, addition of NOHA or **8** led to a significant increase in the rate of NADPH consumption [around 100 nmol min^{-1} (mg of protein)$^{-1}$]. Interestingly, NADPH consumption and nitrite and nitrate formation seem to be relatively well correlated, approximately 1.2 mol of NADPH being consumed to produce 1 mol of NO_2^- and NO_3^-, except in the case of *N*-hydroxyguanidine **7** itself, and of **8**, for which about 3 mol of NADPH was consumed for the formation of 1 mol of NO_2^- and NO_3^- (Table 1).

(3) Organic Products Formed in the BH_4-Free NOS II-Dependent Oxidation of *N*-Hydroxyguanidines. Quantitative measurements of the organic products derived from NOHA and its α-amino acid analogues were found to be very difficult after derivatization of their α-amino acid function (*18*, *30*, *37*). This originated from the relatively low enzymatic activities of BH_4-free NOS II (Table 1) and the great importance of small amounts of products containing a urea function that were formed from hydrolysis of the corresponding cyanamides under the derivatization conditions (*37*).

However, quantitative detection of substrate **8** and of its metabolites was easily performed after HPLC separation and detection with UV spectroscopy at the wave-

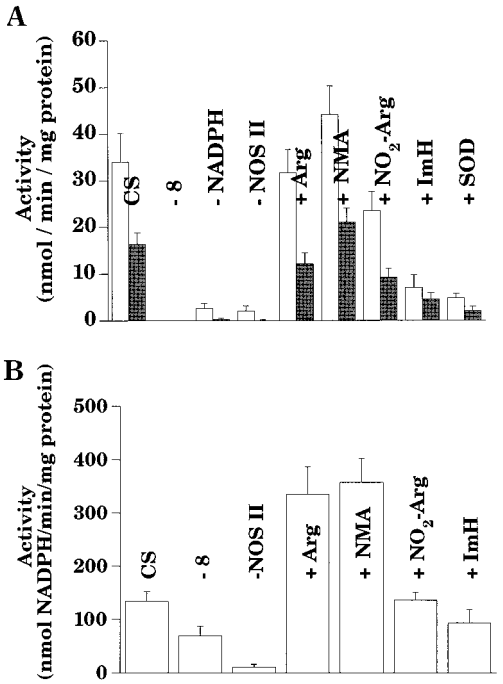
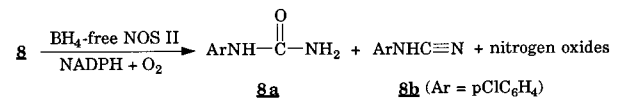


Figure 4. Influence of cofactors, inhibitors, and SOD on urea **8a** and cyanamide **8b** production (A) and NADPH oxidation (B) in oxidation of **8** by BH_4-free NOS II. The control incubation (complete system, CS) was carried out with 500 μM **8**, 1 mM NADPH, and 2.5 μg of NOS II in a total volume of 50 μL. After 5 min at 37 °C, incubations were quenched and submitted to HPLC analysis as described in Materials and Methods. In experiments regarding NADPH consumption, 100 μM NADPH was used. When mentioned, **8**, NADPH, or NOS was omitted and Arg, NMA, NO_2-Arg (1 mM final concentration), or ImH (10 mM final concentration) was added. The SOD concentration was 10 units/mL. Activities for urea **8a** (white bars) and cyanamide **8b** formation (black bars) are means ± SD of four separate experiments.

length corresponding to the absorption of their aryl chromophore. Formation of two products, 4-chlorophenylurea (**8a**) and 4-chlorophenylcyanamide (**8b**), was demonstrated from comparison of their retention times and UV spectra with those of authentic samples.



8a and **8b** were formed with relatively low rates [34 and 16 nmol min^{-1} (mg of protein)$^{-1}$, respectively, for 500 μM **8**] and with a stoichiometry relative to NO_2^- and NO_3^- of ~1 (Table 1). Both products were hardly detectable after incubation in the absence of either NADPH or BH_4-free NOS II. As previously found for NO_2^- formation in BH_4-free NOS II-dependent oxidation of NOHA, addition of Arg or NMA did not significantly inhibit the formation of **8a** and **8b**. Addition of N^{ω}-nitro-L-arginine (NO_2-Arg) resulted in only 40% inhibition of **8a** and **8b** formation, while imidazole inhibited 60% of this activity (Figure 4A). Moreover, even low levels of SOD (1 unit/mL) almost completely inhibited formation of **8a** and **8b** (Figure 3B) as well as formation of NO_2^- (data not shown), whereas oxidation of **8** to **8a** by BH_4-sufficient NOS II was not inhibited by SOD (Figure 3B), as previously reported (*21*).

As in the case of NOHA (Figure 2A,B), **8a** and **8b** formation and NADPH consumption by BH_4-free NOS

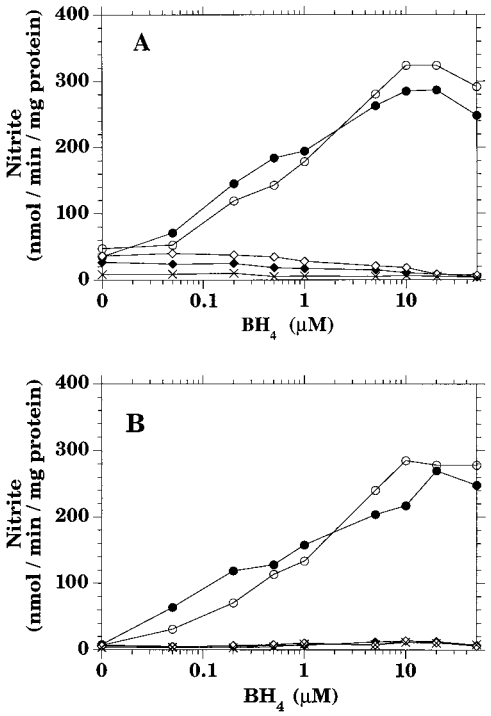


Figure 5. Effect of increasing concentrations of BH_4 on the reaction between NOS II and *N*-hydroxyguanidines **3**−**7** (A) in the absence of SOD and (B) in the presence of 50 units/mL SOD. Incubations were carried out as described in Table 1 except for the presence of BH_4 which was added from a 250 μM solution prepared just before the experiment in 50 mM HEPES (pH 7.4): (●) homo-NOHA, (○) NOHA, (◆) nor-NOHA, (◇) dinor-NOHA, and (×) OH-gua. Means of three separate experiments (SD < 15%).

II in the presence of **8** exhibited similar variations upon addition of modulators of electron transfer. Arg and NMA had little effect or led to an increase in the level of product formation (**8a** and **8b**) and of NADPH consumption (Figure 4A,B); imidazole led to a decrease in these two activities, and NO_2-Arg did not exhibit any significant effect. The strong inhibitory effects of SOD on formation of **8a** and **8b** from oxidation of **8** by BH_4-free NOS II and the parallel variation of **8a** and **8b** formation and NADPH consumption in the presence of NOS regulators are in agreement with an oxidation of **8** by $O_2^{\bullet -}$ derived from the oxidase function of BH_4-free NOS II.

Effects of BH_4 on the Oxidation of *N*-Hydroxyguanidines 3−8 with NOS II. Preincubation of recombinant BH_4-free NOS II with increasing concentrations of BH_4 led to a variation of the rates of NO_2^- formation upon oxidation of *N*-hydroxyguanidines that is greatly dependent on the substrate structure. In fact, two very different kinds of behavior were observed (Figure 5). With NOHA and homo-NOHA as substrates, the rate of NO_2^- (and NO_3^-, data not shown) formation dramatically increased with increasing BH_4 concentration and reaches, with BH_4 concentrations of >10 μM, a maximum level 6−10-fold higher than that observed in the absence of BH_4 (Figure 5A). In the presence of such concentrations of BH_4, SOD did not show any inhibitory effects (Figure 3A). Thus, nitrite formation from BH_4-free NOS II-catalyzed oxidation of NOHA and homo-NOHA is due to oxidation of these compounds by $O_2^{\bullet -}$ derived from the oxidase function of the enzyme, while in the presence of BH_4, it is due to the classical monooxygenase function of NOS II, which occurs in the active site and is not inhibited by SOD. This conclusion was completely con-

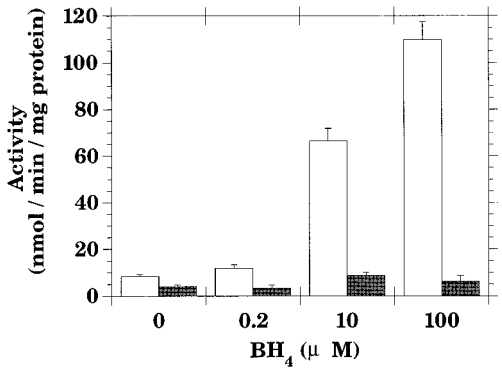


Figure 6. Effect of increasing concentrations of BH_4 on the reaction between NOS II and **8**. Incubations were carried out as described in the legend of Figure 4, except for the addition of BH_4 which was preincubated for 10 min at 4 °C in the presence of NOS II at the indicated concentrations. Reactions were started by the addition of 500 μM **8** and 1 mM NADPH and carried out for 5 min at 37 °C. They were quenched and analyzed by HPLC as described in Materials and Methods to quantify urea **8a** (white bars) and cyanamide **8b** (black bars) formation. Means ± SD of three separate experiments.

firmed by experiments similar to those described in the legend of Figure 5A, except for the presence of 50 units/mL SOD in the incubations (Figure 5B). In the presence of SOD, very similar curves were obtained for the variation of the extent of NO_2^- formation as a function of BH_4 concentration, except that nitrite was hardly detectable in the absence of BH_4.

In the case of the other *N*-hydroxyguanidines, nor-NOHA (**5**), dinor-NOHA (**6**), and hydroxyguanidine itself (**7**), the presence of increasing concentrations of BH_4 failed to produce any increase in the activity. It even led to a slight decrease in the level of NO_2^- formation, which became hardly detectable at BH_4 concentrations of >20 μM (Figure 5A). In experiments performed in the presence of 50 units/mL SOD (Figure 5B), the level of nitrite formation remained close to zero, indicating that the oxidase activity of NOS II is the only activity responsible for the oxidation of **5**−**7**, whatever the concentration of BH_4 (compare panels A and B of Figure 5).

Compound **8**, which has been recently described to act as an exogenous substrate of NOS II with formation of stoichiometric amounts of NO and urea **8a** (*21*), exhibited a behavior similar to those of NOHA and homo-NOHA. For instance, Figure 6 shows that, in the presence of increasing concentrations of BH_4, the rate of formation of urea **8a** was dramatically increased, by a factor of at least 10, whereas that of cyanamide **8b** was only slightly modified. As in the case of NOHA and homo-NOHA, oxidation of **8** with BH_4-free NOS II was almost suppressed in the presence of SOD (Figure 3B), whereas its oxidation with BH_4-containing NOS II was not affected by the presence of SOD (Figure 3B).

Discussion

In the absence of BH_4, NOS II is not able to catalyze the oxidation of compounds containing a guanidine function, such as Arg and homo-Arg, as shown by the lack of any formation of NO_2^- or NO_3^- under the conditions used here (Table 1). However, BH_4-free NOS II does catalyze the oxidation of a wide range of compounds involving an *N*-hydroxyguanidine function, **3**−**8**, with similar rates of NO_2^- and NO_3^- formation, if one excepts a markedly lower rate in the particular case of *N*-hydroxyguanidine **7** itself (Table 1). The main characteristics of these reactions, i.e., (i) their almost complete inhibition by very low concentrations of SOD, (ii) their relatively low rates [20−80 nmol min^{-1} (mg of protein)$^{-1}$ at 500 μM substrate], (iii) their broad substrate specificity, (iv) their poor chemoselectivity, as shown by the formation of amounts of urea **8a** and cyanamide **8b** in a 2/1 ratio in the case of **8**, are very similar to those previously reported for the oxidations of *N*-hydroxyguanidines with microsomal cytochromes P450 (*38*). These results show that BH_4-free NOS II, like cytochromes P450, oxidizes *N*-hydroxyguanidines via $O_2^{\bullet -}$ derived from the oxidase function of these two classes of heme proteins. Accordingly, the aforementioned characteristics of these reactions are very similar to those reported for the chemical oxidation of *N*-hydroxyguanidines by $O_2^{\bullet -}$ itself in aqueous or organic solvents (*38*, *41*−*44*). Thus, the ability of $O_2^{\bullet -}$ to perform the oxidative cleavage of the C=NOH function of various *N*-hydroxyguanidines, whereas it seems to be inert toward guanidines (*43*), allows one to understand the broad substrate specificity of BH_4-free NOS II toward *N*-hydroxyguanidine and its inertness toward Arg (ref *30* and this work) and homo-Arg (Table 1).

As mentioned above, Arg and NMA, which were expected to compete with the *N*-hydroxyguanidine substrate for binding to the active site, failed to inhibit the oxidation of NOHA and **8** with BH_4-free NOS II (Figures 2 and 4). This result suggests that the reaction between these *N*-hydroxyguanidines and $O_2^{\bullet -}$ does not occur in the active site but rather in the surrounding buffer. Therefore, the only ways to prevent substrate oxidation with NO_2^- formation are inhibition of superoxide synthesis with inhibitors of the electron transfer to the heme such as imidazole, NO_2-Arg, and TC (Figures 2A and 4A) and scavenging of the freshly produced superoxide by SOD. Diffusion of $O_2^{\bullet -}$ toward the solvent buffer also explains why total inhibition of its reaction with the studied *N*-hydroxyguanidines can be achieved with very small quantities of SOD.

Thus, oxidation of *N*-hydroxyguanidines by BH_4-free NOS II probably decomposes as follows:

$$O_2 + 0.5\ NADPH \xrightarrow{NOS} O_2^{\bullet -} + 0.5\ NADP^+ + 0.5\ H^+$$

$$O_2^{\bullet -} + RNH{-}C(=NOH){-}NH_2 \longrightarrow RNH{-}C(=O){-}NH_2 + RNHCN + NO_2^- \text{ and other nitrogen oxides}$$

The first step is totally NOS-dependent; however, some *N*-hydroxyguanidines may exert a subtle control on this step as shown in Table 1 in the case of NOHA. On the contrary, the yield and rate of the second step will only depend on the reactivity of the *N*-hydroxyguanidine toward $O_2^{\bullet -}$, which is based on the structural and electronic characteristics of the C=NOH function, as was demonstrated in the case of P450-dependent oxidation of *N*-hydroxyguanidines (*38*).

Addition of BH_4 to recombinant BH_4-free NOS II dramatically changes the behavior of this enzyme toward *N*-hydroxyguanidines, by playing two key roles as shown in Figures 5A and 6. The first role is to decrease the importance of the $O_2^{\bullet -}$-dependent oxidation of *N*-hydroxyguanidines. This is shown by the progressive decrease in the level of and final complete loss of NO_2^- formation from the oxidation of *N*-hydroxyguanidines **5**−**7** that are

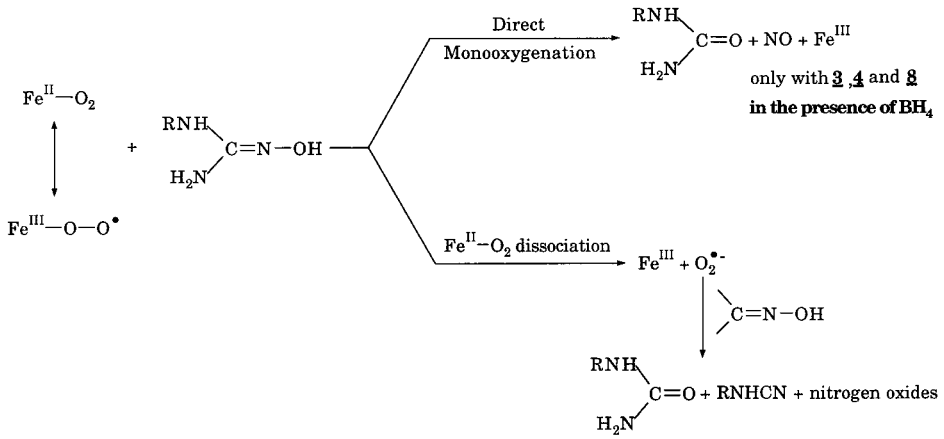


Figure 7. Possible evolutions of the NOS complex [$Fe^{II}—O_2 \leftrightarrow Fe^{III}—OO^{\bullet}$] in the presence of N^{ω}-hydroxyguanidines.

not substrates of BH_4-containing NOS II (*18*) (Figure 5A). This aptitude of BH_4 to strongly decrease the level of formation of $O_2^{\bullet-}$ derived from the NOS oxidase function has been reported for the three main NOS isozymes (*23*, *45*−*47*). The detailed molecular origin of this effect remains to be determined.

The second role of BH_4 in NOS II-dependent oxidation of *N*-hydroxyguanidines is more spectacular (Figures 5A and 6). It is to start a new monooxygenase activity which only occurs with substrates of BH_4-containing NOS II able to produce NO, such as NOHA, homo-NOHA, and **8**. The characteristics of this reaction are completely different from those of the BH_4-free NOS II reactions, and correspond well to those previously described for NOS II-dependent monooxygenation of Arg, NOHA, homo-NOHA, and **8** (*3*, *18*, *21*). They include (i) a weak effect of SOD on the activity, (ii) a clear substrate selectivity as compounds **5**−**7** are not transformed, (iii) much higher activity levels [between 100 and 320 nmol min^{-1} (mg of protein)$^{-1}$], and (iv) a high chemoselectivity with the almost exclusive formation of urea **8a** from **8** (Figure 6). Moreover, this activity is strongly inhibited by compounds known to bind at the active site of NOS, such as Arg (*16*, *17*). Therefore, this monooxygenase reaction is performed inside the active site presumably by the NOS $Fe^{II}—O_2$ species (*8*−*12*).

Another important difference between the oxidations catalyzed either by BH_4-containing NOS II or by BH_4-free NOS II is the clear formation of NO, in a NO/urea ratio close to 1, in the former reactions (*4*, *5*, *18*, *21*) contrary to the very low level of formation of NO in the latter reactions. In fact, as mentioned above in Results, oxidation of compounds **1**−**8** by BH_4-free NOS II did not lead to any significant detection of NO by the oxyhemoglobin assay. On the other hand, oxidation of NOHA, homo-NOHA, and **8** by BH_4-containing NOS II has been reported to produce NO in stoichiometric amounts relative to the corresponding urea (*18*, *21*).

The different behavior exhibited by BH_4-free and BH_4-containing NOS II toward *N*-hydroxyguanidines could be interpreted with the different possible fates of the NOS $Fe^{II}—O_2$ intermediate (Figure 7). This species may either directly react with the C═NOH function of *N*-hydroxyguanidine substrates such as NOHA or dissociate its $Fe^{II}—O_2$ bond, leading to NOS Fe^{III} and $O_2^{\bullet-}$. Because of the relatively weak oxidizing properties of heme $Fe^{II}—O_2$ complexes (*48*), it is likely that it should rapidly react only with especially reactive substrates very well positioned close to it in the NOS active site. Such a fast reaction of the NOS $Fe^{II}—O_2$ intermediate with the chemically reactive C═NOH function of *N*-hydroxyguanidines appears to occur only with compounds well recognized and bound close to the heme, such as NOHA, homo-NOHA, and **8**, when BH_4 is present. In all the other situations, i.e., with the other *N*-hydroxyguanidines **5**−**7** in the presence or in the absence of BH_4, and with all the *N*-hydroxyguanidines in the absence of BH_4, the NOS $Fe^{II}—O_2$ species does not react with the C═NOH function. It thus dissociates its $Fe^{II}—O_2$ bond, and all the products that have been observed come from reactions of $O_2^{\bullet-}$ with the *N*-hydroxyguanidines. In that respect, it is noteworthy that $O_2^{\bullet-}$ might come not only from dissociation of the NOS $Fe^{II}—O_2$ complex but also from reduction of O_2 at the level of the NOS reductase domain (*23*, *46*, *49*−*51*). However, the 80% inhibition of BH_4-free NOS II-dependent oxidation of **8** by 10 mM imidazole, a known NOS−iron ligand (Figure 4A), indicates that only a small part of $O_2^{\bullet-}$ directly comes from the reductase domain under the conditions that were used.

One easily understands the requirement of a strict proximity between the substrate C═NOH function and the NOS $Fe^{II}—O_2$ intermediate for a fast enough reaction of the poorly reactive $Fe^{II}—O_2$ species, which explains why only some *N*-hydroxyguanidines undergo efficient monooxygenation. The absolute requirement of BH_4 for this reaction could be explained, at least in part, by its role in favoring a good positioning of the C═NOH function of NOHA and related substrates in proximity of the $Fe^{II}—O_2$ species. Accordingly, BH_4 is known to play a key role in the formation of active NOS II dimers (*12*), and recent X-ray data about NOSs have shown the presence of a network of hydrogen bonds between BH_4, heme, and Arg (*16*, *17*) that should be crucial to controlling the proximity between the substrate and heme $Fe^{II}—O_2$ species. BH_4 could also favor the reaction between the substrate C═NOH function and heme $Fe^{II}—O_2$ species by increasing the intrinsic reactivity of the $Fe^{II}—O_2$ species (*14*), or by transferring electron density to the heme (*15*, *16*). Moreover, evidence for electron transfer from BH_4 to the heme of the NOS $Fe^{II}—O_2$ species has been provided very recently (*52*, *53*).

In conclusion, the aforementioned results show that NOS II and BH_4-free NOS II are able to catalyze the oxidation of many *N*-hydroxyguanidines with formation of the corresponding ureas and cyanamides and nitrogen oxides. Most of these reactions are performed by $O_2^{\bullet-}$

derived from the oxidase function of NOS II. Only few of them are selective monooxygenations performed by the NOS $Fe^{II}-O_2$ species, which exclusively occur with NOHA, homo-NOHA, and **8**, when BH_4 is present. These results suggest a possible implication of NOSs in the oxidative metabolism of certain classes of xenobiotics not only via their monooxygenase function, as previously mentioned (case of **8**; *21*) but also via their oxidase function. In fact, oxidation of exogenous *N*-hydroxyguanidines, which are not substrates of the NOS monooxygenase function and which lead to easily detected ureas or cyanamides because of the NOS oxidase function, could be used to evaluate $O_2^{\bullet-}$ formation by NOS II. Finally, our results provide a further clear illustration of the key role of BH_4 in regulating the monooxygenase/oxidase ratio in NOS (*23*, *29*, *39*, *45*−*47*).

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