Multiple Catalytic Functions of Brain Nitric Oxide Synthase

BIOCHEMICAL CHARACTERIZATION, COFACTOR-REQUIREMENT, AND THE ROLE OF N'-HYDROXY-L-ARGININE AS AN INTERMEDIATE*

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Brain NO (nitric oxide) synthase contains FAD, FMN, heme, and tetrahydrobiopterin as prosthetic groups and represents a multi-functional oxidoreductase catalyzing oxidation of L-arginine to NO and L-citrulline, formation of H₂O₂, and reduction of cytochrome c. We show that substrate analogues and inhibitors interacting with the heme block both the reductive activation of oxygen and the oxidation of L-arginine without affecting cytochrome c reduction. We further demonstrate that N'-hydroxy-L-arginine is an intermediate in enzymatic NO synthesis. The ratio of L-citrulline to free N'-hydroxy-L-arginine was ≥50 under various assay conditions, but could markedly be reduced down to 4 by redox active inhibitors. Brain NO synthase is shown to utilize both L-arginine and N'-hydroxy-L-arginine for the formation of stoichiometric amounts of NO and L-citrulline. Tetrahydrobiopterin equally enhanced reaction rates from either substrate (~5-fold), but its rate accelerating effects were only observed at NADPH concentrations ≥3 μM. In the absence of L-arginine or tetrahydrobiopterin, brain NO synthase catalyzes the generation of H₂O₂. We now show that, in contrast to L-arginine, N'-hydroxy-L-arginine fully blocked H₂O₂ formation in the absence of exogenous tetrahydrobiopterin, indicating that N'-hydroxy-L-arginine is a direct inhibitor of enzymatic oxygen activation. Based on these data, a hypothetical mechanism of enzymatic NO formation is discussed.

The inter- and intracellular messenger molecule nitric oxide (NO) is involved in a variety of important physiological processes like vascular relaxation, platelet activation, neurotransmission, and unspecific immune response (1–3). NO is formed from L-arginine by different NO synthase (NOS)1 isozymes which differ in their subcellular distribution and in the mechanisms of their regulation (4). Until now, three major NOS isoforms have been purified and cloned: a Ca²⁺/calmodulin-dependent, cytosolic enzyme from brain (5–8), a cytosolic enzyme inducible, Ca²⁺-independent NOS from activated macrophages (9–13), and a membrane-associated isoform, which is also Ca²⁺-dependent, from vascular endothelial cells (14–16).

Biochemical characterization of brain and macrophage NOS demonstrated that both isoforms contain FAD, FMN, and heme as prosthetic groups (10, 11, 17–23). Furthermore, all isoforms described so far are stimulated by tetrahydrobiopterin (H₂biopterin) (6, 14, 24–28), and it was shown that some amounts of H₂biopterin remain tightly bound to brain and macrophage NOS during enzyme purification (17, 19, 28).

These different NOS isoforms catalyze an NADPH-dependent conversion of L-arginine into L-citrulline and NO. The mechanism of this reaction is still incompletely understood. It is known that the nitrogen in the NO molecule derives from the guanido group of L-arginine (29–32) and that molecular oxygen is incorporated into both NO and L-citrulline (33, 34). Early suggestions that NOHLA may be an intermediate in this reaction (29, 30) are supported by recent results obtained by Stuehr and coworkers (35) with inducible NOS purified from activated macrophages. Since this group reported that conversion of [³H]arginine into the labeled intermediate was only detectable under very special conditions, i.e. in the presence of high concentrations of unlabeled NOHLA, the putative intermediate may normally not be released from the enzyme. Other groups have used crude subcellular fractions or intact cells to demonstrate that endothelial NOS also utilizes NOHLA for NO synthesis (34, 36).

Brain NOS catalyzes a rapid NADPH- and Ca²⁺/calmodulin-dependent reduction of cytochrome c, which is apparently due to a direct interaction of the cytochrome with NOS (37). Together with the findings that the enzyme exhibits pronounced sequence similarities to cytochrome P-450 reductase (8) and contains FAD, FMN, and heme (17–23), these data indicate that a flavin-mediated electron transport from NADPH to enzyme-bound heme is a crucial step in NO synthesis. This electron transport also operates in the absence of substrate, but superoxide anion and H₂O₂ are produced instead of NO and L-citrulline under these conditions (38, 39). The rates of H₂O₂ and L-citrulline formation were found to be inversely correlated, as the complete coupling of oxygen reduction to NO formation did not only require saturating concentrations of L-arginine but also exogenously added H₂biopterin (38).

In the present study we have characterized the various enzymatic activities of purified brain NOS, in order to assign them to the different prosthetic groups of the enzyme. We especially focused on the role of NOHLA as an intermediate in enzymatic NO formation and investigated the redox reactions involved in the formation and cleavage of this compound. For this purpose we settled the cofactor requirements of NOS with both substrates and screened for redox-active
inhibitors specifically interfering with the cleavage of the intermediate. Based on our findings that L-arginine and NOHLA exhibited different effects on NOS-catalyzed oxygen activation we have worked out a hypothetical reaction scheme of substrate oxidation at the heme site.

EXPERIMENTAL PROCEDURES

Materials

NOS was purified from porcine brain as previously described (6, 17). For reducing the concentration of NADPH, some preparations (1-2 ml each; 0.3-0.4 mg/ml) were dialyzed for 24 h against three changes of 1 liter of a triethanolamine/HCl buffer (50 mM, pH 7.0) containing 0.5 mM EDTA without substantial loss of enzyme activity. N-\textsuperscript{\textdegree}Hydroxy-L-arginine and N-\textsuperscript{\textdegree}hydroxy-L-[2,3-\textsuperscript{\textit{H}}]arginine were synthesized according to Ref. 40. All other \textsuperscript{\textit{H}}-labeled compounds were obtained from DuPont de Nemours, Dreieich, Federal Republic of Germany. L-[2,3-\textsuperscript{\textit{H}}]arginine and N-\textsuperscript{\textdegree}hydroxy-L-[2,3-\textsuperscript{\textit{H}}]arginine were further purified by HPLC on a cation exchange column (Nucleosil 100-10 SA, 250 x 8 x 4 mm, 10-\mu m pore size, Macherey & Nagel, Düren, F.R.G.) using an aqueous solution of 50 mM sodium acetate, pH 6.5, at a flow rate of 1.5 ml/min as eluant. All other reagents were obtained from Sigma, F.R.G., or from sources described previously (6, 17, 37, 38).

Methods

Determination of NOS Activity—NOS activity was determined as formation of NO and L-citrulline from L-arginine or NOHLA. Enzymatic NO formation was measured photometrically as NO-induced loss of oxyhemoglobin (41) as previously described (42). Rates of L-[2,3-\textsuperscript{\textit{H}}]citrulline formation from L-[2,3-\textsuperscript{\textit{H}}]arginine or N-\textsuperscript{\textdegree}hydroxy-L-[2,3-\textsuperscript{\textit{H}}]arginine (10,000-15,000 disintegrations/min/nmol) were determined as described (43). Purified brain NOS (0.3-0.6 mg/ml) was incubated at 37 °C in final volumes of 0.1 ml (L-citrulline formation) or 0.2 ml (NO formation) of a triethanolamine/HCl buffer (50 mM, pH 7.0). Unless otherwise indicated, reaction mixtures contained 0.1 mM L-arginine or NOHLA, 10 pg/ml calmodulin, 3 \mu M free Ca\textsuperscript{2+}, and 0.1 mM NADPH at 37 °C. For the determination of NOS activities, the method was calibrated by carrying out reaction procedures in the absence of added enzyme.

Calcium-dependent NADPH oxidation and NADPH:cytochrome c oxidoreductase activities of NOS were determined in the absence of substrate as described previously (17, 37). Unless otherwise indicated, purified NOS (1-2 \mu g) was incubated in final volumes of 0.2 ml of a 50 mM triethanolamine/HCl buffer, pH 7.0, in the presence of 3 \mu M free Ca\textsuperscript{2+}, 10 \mu g/ml calmodulin, and 0.1 mM NADPH at 37 °C. For the determination of cytochrome c oxidoreductase activity of NOS, 0.2 mM cytochrome c was additionally present. The absorbance increase at 340 nm (NADPH) or 550 nm (cytochrome c) were continuously monitored against calcium-deficient blank samples. Rates of NADPH oxidation and cytochrome c reduction were calculated using extinction coefficients of 6.34 and 21 mm\textsuperscript{-1} x cm\textsuperscript{-1}, respectively.

H\textsubscript{2}O\textsubscript{2} generation was measured by two independent methods, as formation of ferric thiocyanate (38) and as luminol-dependent chemiluminescence (46). Unless otherwise indicated, purified NOS (1-2 \mu g) was incubated at 37 °C for 10 min in 0.2 ml of a 50 mM triethanolamine/HCl buffer, pH 7.0, containing 0.1 mM NADPH, 3 \mu M free Ca\textsuperscript{2+}, and 10 \mu g/ml calmodulin. For the chemiluminescence assay, 50-\mu l aliquots were removed, and reactions were terminated by the addition of 450 \mu l of a 50 mM sodium phosphate buffer, pH 7.0, containing 2.5 mM EDTA. 50-\mu l aliquots (50 \mu l) were further diluted 1:15 with this buffer and assayed for luminol-dependent chemiluminescence in the presence of 10 \mu M luminol, 10 units/ml superoxide dismutase, and 10 units/ml horseradish peroxidase using a Berthold Clinilumat LB 3502 (Berthold, Vienna, Austria). Control incubations were run in the absence of calmodulin, and the method was calibrated by carrying out authentic H\textsubscript{2}O\textsubscript{2} through the incubation procedures.

HPLC Analysis of L-Arginine Metabolites—Unless otherwise indicated, purified NOS (0.5-1 \mu g) was incubated at 37 °C in a final volume of 0.1 ml of a triethanolamine/HCl buffer (50 mM, pH 7.0), containing 10 \mu M L-[2,3-\textsuperscript{\textit{H}}]arginine (100,000 disintegrations/min/nmol), 10 \mu g/ml of calmodulin, 3 \mu M free Ca\textsuperscript{2+}, 0.1 mM NADPH, 5 \mu M FAD, 5 \mu M FMN, and 10 \mu M H\textsubscript{4}bioppterin. Blank values were determined in the absence of added enzyme.

When the generation of NO was monitored photometrically as loss of oxyhemoglobin, similarly, formation of L-citrulline from either substrate also followed Michaelis-Menten kinetics (not shown). Table I summarizes the obtained enzyme kinetic parameters.

![Graph](https://via.placeholder.com/150)

**Fig. 1. Eadie-Hofstee plots of NOS-catalyzed NO formation from L-arginine and NOHLA.** Purified NOS (0.3-0.6 \mu g/ml) was incubated at 37 °C in 0.2 ml of a triethanolamine/HCl buffer (50 mM, pH 7.0), containing 10 \mu g/ml calmodulin, 3 \mu M free Ca\textsuperscript{2+}, 0.1 mM NADPH, 5 \mu M FAD, 5 \mu M FMN, and 10 \mu M H\textsubscript{4}bioppterin in the presence of increasing concentrations of L-arginine (solid circles) or NOHLA (open circles). Rates of NO formation were quantified using the oxyhemoglobin assay described under “Experimental Procedures.” The plot shown is representative of three.

**TABLE I**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction product</th>
<th>K\textsubscript{m} (\mu M)</th>
<th>V\textsubscript{max} (\textmu mol x mg\textsuperscript{-1} x min\textsuperscript{-1})</th>
</tr>
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<tbody>
<tr>
<td>L-Arginine</td>
<td>NO</td>
<td>10 (7-14)</td>
<td>0.57 ± 0.06</td>
</tr>
<tr>
<td>NOHLA</td>
<td>L-Citrulline</td>
<td>10 (5-17)</td>
<td>0.54 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23 (17-30)</td>
<td>0.58 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 (19-48)</td>
<td>0.57 ± 0.03</td>
</tr>
</tbody>
</table>

**Characterization of Brain Nitric Oxide Synthase**
parameters. Stoichiometrical amounts of NO and L-citrulline were produced from both substrates, and maximal reaction rates were 0.6 μmol × mg⁻¹ × min⁻¹. The Kᵣ values for NOHHLA were about 2-3 fold higher than those obtained with L-arginine as substrate: 10 versus 23 μM and 10 versus 30 μM when assayed as formation of NO and L-citrulline, respectively. 

To find out whether NOHHLA is an intermediate in NO synthesis, purified brain NOS was incubated with L-[2,3-³H]arginine and radioactively labeled metabolites were separated by HPLC. As demonstrated by the chromatogram shown in Fig. 2, L-citrulline and NOHHLA were the sole labeled L-arginine metabolites detectable. The relative amount of NOHHLA produced during the incubation was quantified as the molar ratio of L-citrulline to NOHHLA. Under control conditions, these ratios were ≥50, indicating that NOHHLA is an intermediate in NO synthesis, but is not released from the enzyme in considerable amounts during the reaction. It has been suggested that release of the intermediate is affected by cofactor concentrations (35). We did not observe any accumulation of NOHHLA, neither in the absence of H₄bioprotein nor by varying the concentrations of NADPH (from 1 to 100 μM) in the presence of 10 μM H₄bioprotein. It has further been proposed that two distinct reaction steps, i.e. formation of NOHHLA and subsequent oxidative cleavage of the intermediate, are involved in NO synthesis (17, 18, 30, 35). We speculated that redox-active inhibitors of NOS may preferentially interfere with one of these two reactions and determined L-citrulline/NOHHLA ratios in the presence of various enzyme inhibitors. The substrate analogues L-NMA (0.1 mM) and L-NNA (0.03 mM) did not change the relative amount of NOHHLA formation, but the redox-active NOS inhibitors nitro blue tetrazolium (0.1 mM) and methylene blue (0.03 mM) (19, 37, 46) considerably decreased the L-citrulline/NOHHLA ratios from ≥50 to 6 and 4, respectively.

It has been suggested that the NOS cofactor H₄bioprotein is required for the monooxygenase-like hydroxylation of L-arginine (17, 25), Results obtained previously with macrophage NOS, however, indicate that NO synthesis from both L-arginine and NOHHLA is stimulated by H₄bioprotein (35). We recorded H₄bioprotein concentration-response curves with both substrates to settle this issue with the brain enzyme. As shown in Fig. 3, H₄bioprotein enhanced the formation of NO and L-citrulline from either L-arginine (panel A) or NOHHLA (panel B) approximately 5-fold with similar EC₅₀ values (0.2 and 0.3 μM with L-arginine and NOHHLA as substrates, respectively). Further experiments revealed an interaction of H₄bioprotein and NADPH during NO synthesis. Fig. 4 shows NADPH concentration-response curves recorded in the absence (open symbols) and presence (solid symbols) of H₄bioprotein. The rate-accelerating effects of H₄bioprotein were largely dependent on the concentration of NADPH. No great effects of H₄bioprotein were observed at NADPH concentrations ≤3 μM, whereas NOS activity was enhanced 4-5-fold by H₄bioprotein in the presence of ≥0.1 mM NADPH. Similar results were obtained with NOHHLA as a substrate (data not shown).
In addition to the formation of NO and L-citrulline, brain NOS catalyzes a reduction of cytochrome c (37) as well as a substrate-independent oxidation of NADPH (17), which leads to the formation of H$_2$O$_2$ (38), with superoxide being produced as an intermediate (39). We determined the potencies of NADPH and calmodulin as cofactors of these multiple catalytic functions of NOS. As shown in Table II, we did not detect any significant differences in the cofactor dependences of the various reactions. The half-maximally effective concentrations of NADPH and calmodulin ranged from 8-19 and 30-62 nM, respectively. Thus, L-citrulline formation from either L-arginine or NOHLA, substrate-independent oxidation of NADPH and reduction of cytochrome c apparently share common binding sites for NADPH and calmodulin.

In order to assign the multiple catalytic functions of brain NOS to its different prosthetic groups, we compared the effects of selected inhibitors on the formation of L-citrulline, on H$_2$O$_2$ generation, and on the reduction of cytochrome c. In addition to the well established substrate analogues L-NNA and L-NMA, miconazole and KCN were used as inhibitors of heme-catalyzed oxygenase reactions. As shown in Table III, conversion of 0.1 mM L-arginine into L-citrulline was inhibited by L-NNA, L-NMA, miconazole, and KCN with half-maximally effective concentrations of 1.1 μM, 10 μM, 94 μM and 4.2 mM, respectively. L-Citrulline formation from NOHLA was similarly affected by these compounds (data not shown). L-NNA also blocked H$_2$O$_2$ formation (IC$_{50}$ = 0.20 μM), whereas L-NMA did not affect this reaction in concentrations up to 1 mM. Generation of H$_2$O$_2$ was inhibited by miconazole and KCN with IC$_{50}$ values of 50 μM and 9.7 mM, respectively. Cytochrome c reduction, however, was neither affected by N$_2$-substituted substrate analogues nor by miconazole or KCN.

The effects of L-arginine on NOS-catalyzed H$_2$O$_2$ formation were compared with those of the putative intermediate NOHLA. To make the intriguing results we obtained as reliable as possible, H$_2$O$_2$ was assayed by two independent methods (see “Experimental Procedures”) which yielded closely similar results (not shown). As illustrated in Fig. 5A, L-arginine completely inhibited H$_2$O$_2$ formation only in the presence of exogenously added H$_4$biopterin (10 μM). Omission of the cofactor (open circles) led to much less pronounced effects of L-arginine (~30% inhibition at 1 mM), confirming our previous suggestion that its inhibitory effect is quantitatively correlated to the rates of NO formation (38). Rather different results were obtained, however, with NOHLA. The intermediate fully inhibited H$_2$O$_2$ formation even in the absence of exogenous H$_4$biopterin, and its inhibitory potency was not significantly enhanced by addition of 10 μM H$_2$biopterin (Fig. 5B).

As an apparently direct inhibitor of NOS-catalyzed oxygen activation, NOHLA resembles the substrate analogue L-NNA but while NOHLA serves as a substrate, L-NNA was described as an irreversible inhibitor of the brain enzyme (47). We investigated whether inhibition of purified brain NOS by L-NNA is a time- and calmodulin-dependent effect. As shown in Fig. 6, formation of L-citrulline from 0.1 mM L-arginine was decreased by 10 μM L-NNA in a time-dependent manner from 69 (after 30 s) to 11% (after 10 min) of the respective control samples incubated in the absence of the inhibitor. Enzyme inactivation was more rapid when NOHLA (0.1 mM) was present instead of L-arginine and occurred almost immediately when the oxidation of NADPH was monitored in the absence of either substrate (see Fig. 6). To find out whether enzymatic conversion of L-NNA accounts for the observed inactivation process, we preincubated NOS with 1 μM L-NNA in the absence and presence of calmodulin prior to the determination of enzymatic activity. For these experiments, incubation times were reduced to 0.5 min in order to minimize inactivation during the assay. As shown in Table IV, 1-

### Table II

<table>
<thead>
<tr>
<th>NO synthase activity</th>
<th>EC$_{50}$ values</th>
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<tbody>
<tr>
<td></td>
<td>NADPH</td>
</tr>
<tr>
<td>----------------------</td>
<td>--------</td>
</tr>
<tr>
<td>L-Citrulline formation</td>
<td>µM</td>
</tr>
<tr>
<td>From L-Arginine</td>
<td>16 (9-22)</td>
</tr>
<tr>
<td>From NOHLA</td>
<td>8 (5-16)</td>
</tr>
<tr>
<td>NADPH oxidation</td>
<td>15 (8-26)</td>
</tr>
<tr>
<td>Cytochrome c reduction</td>
<td>19 (14-26)</td>
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### Table III

<table>
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<tr>
<th>Inhibitor</th>
<th>IC$_{50}$ values</th>
</tr>
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<tr>
<td></td>
<td>NADPH oxidation</td>
</tr>
<tr>
<td>L-NNA</td>
<td>1.1 µM (0.7-1.6)</td>
</tr>
<tr>
<td>L-NMA</td>
<td>10 µM (5-21)</td>
</tr>
<tr>
<td>Miconazole</td>
<td>94 µM (73-121)</td>
</tr>
<tr>
<td>KCN</td>
<td>4.2 mM (3.8-4.7)</td>
</tr>
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*NI, not inhibited.*

![Fig. 5](http://example.com/figures/fig5.png)

**Fig. 5. Effects of L-arginine and NOHLA on NOS-catalyzed hydrogen peroxide formation.** Purified NOS (1-2 μg) was incubated for 10 min at 37°C in 0.2 ml of a triethanolamine/HCl buffer (50 mM, pH 7.0), containing 0.1 mM NADPH, 3 mM free Ca$^{2+}$, and 10 μM/ml calmodulin in the presence of increasing concentrations of L-arginine (panel A) or NOHLA (panel B). Reactions were carried out in the absence (open symbols) or presence (solid symbols) of 10 μM H$_4$biopterin. Rates of enzymatic hydrogen peroxide formation were determined using luminal-dependent chemiluminescence as described under “Experimental Procedures.” Results are expressed as percent of control activities determined in the absence of L-arginine or NOHLA. Data are means ± S.E. of three separate experiments.
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Fig. 6. Time-dependent inactivation of brain NOS by L-NNA. Purified NOS (2–3 μg) was incubated with 10 μM L-NNA in the presence of 0.1 mM L-arginine (open circles), 0.1 mM NOHLA (solid circles), and, for the measurement of NADPH oxidation, in the absence of substrate (open squares) at 37°C in 1 ml of a triethanolamine/HCl buffer (50 mM, pH 7.0), containing 10 μg/ml calmodulin, 5 μM free Ca²⁺, 0.1 mM NADPH, 5 μM FAD, 5 μM FMN, and 10 μM H₄biopterin. For the determination of L-citrulline formation (see “Experimental Procedures”), aliquots (0.1 ml) were removed after the times indicated on the abscissa. Oxidation of NADPH was monitored continuously as decrease in absorbance at 340 nm. Data are given as percent of enzymatic activities determined in the absence of L-NNA and are means of three separate experiments.

TABLE IV
Effects of calmodulin and L-arginine on the inactivation of brain NOS by L-NNA

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>NOS activity (μM)</th>
</tr>
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<tbody>
<tr>
<td>- Calmodulin</td>
<td>6 ± 3.8</td>
</tr>
<tr>
<td>+ L-Arginine (0.01 mM)</td>
<td>52 ± 5.9</td>
</tr>
<tr>
<td>+ L-Arginine (0.1 mM)</td>
<td>83 ± 7.7</td>
</tr>
<tr>
<td>+ Calmodulin (10 μg/ml)</td>
<td>3 ± 2.2</td>
</tr>
<tr>
<td>% of control</td>
<td></td>
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</table>

citrulline formation was 6 ± 3.8% of those samples which had been preincubated in the absence of L-NNA. The observed loss of NOS activity was similar in the presence of calmodulin (6 ± 2.2%), and inactivation was attenuated to 52 ± 5.9 and 83 ± 7.7% when L-arginine was included during the preincubations at concentrations of 0.01 and 0.1 mM, respectively.

**DISCUSSION**

Nitric oxide synthase has at least two distinct catalytic domains, an FAD- and FMN-containing reductase domain, and an oxygenase domain that contains ferroprotoporphyrin IX (17–23). In addition to the oxidation of L-arginine, the brain enzyme catalyzes two substrate-independent reactions, *i.e.* reduction of cytochrome c (37) and reduction of molecular oxygen to H₂O₂ (38). We found that KCN and miconazole, which both interfere with heme-dependent reactions, inhibited oxidation of L-arginine and formation of H₂O₂, whereas the reduction of the exogenously added electron acceptor cytochrome c was not affected in the presence of these compounds (see Table III). These data indicate that both activation of molecular oxygen and L-arginine oxidation occur at the heme center of the catalytic site of the enzyme, whereby the required electrons may be provided by the intramolecularly attached flavin-containing reductase domain, which is not affected by the enzyme inhibitors investigated. The almost identical cofactor dependences of the various enzymatic reactions (see Table II) indicate that both domains share common binding sites for NADPH and calmodulin.

Stoichiometric amounts of L-citrulline and NO were generated from L-arginine and NOHLA. Reaction rates were comparable with both substrates, but the apparent Kₘ for L-arginine was lower than that for NOHLA. HPLC analysis of ²H-labeled metabolites of L-arginine revealed that NOHLA was produced as intermediate in the course of NO synthesis. At optimal reaction conditions, the L-citrulline/NOHLA ratios were ≥50, indicating that the intermediate is normally not released from the enzyme but immediately further converted to L-citrulline and NO. Relative NOHLA formation was considerably enhanced in the presence of nitro blue tetrazolium or methylene blue, two reoxidative inhibitors of brain NOS, but not by L-NNA and L-NMA. Nitro blue tetrazolium and methylene blue seem to preferentially interfere with a redox reaction which (i) occurs immediately subsequent to the formation of NOHLA and (ii) is characterized by an electrochemical gradient differing from the redox steps involved in the formation of NOHLA (see below).

Meanwhile it is well established that H₄biopterin represents a cofactor in the biosynthesis of NO (6, 14, 17, 19, 24–28, 48). Since the pteridine is required for the monoxygenase-like hydroxylation of aromatic amino acids (49), it has been proposed that it may act similarly in NO synthesis, being the electron donor for the N-hydroxylation of L-arginine (17, 25). Meanwhile it is well established that H₄biopterin represents a cofactor in the biosynthesis of NO (6, 14, 17, 19, 24–28, 48). Since the pteridine is required for the monoxygenase-like hydroxylation of aromatic amino acids (49), it has been proposed that it may act similarly in NO synthesis, being the electron donor for the N-hydroxylation of L-arginine (17, 25). Their conclusions were predominantly based on their findings that exogenously added H₄biopterin was substoichiometrically active although it was not recycled during the reaction (17, 48). They did not consider, however, the possibility that endogenous H₄biopterin may also be necessary for this first reaction, since we found that neither omission of the pteridine nor the presence of various amounts of NADPH or H₄biopterin gave rise to an accumulation of NOHLA. It is not clear yet how H₄biopterin affects NOS activities. Giovaneli and co-workers suggested that the pteridine may act as an allosteric cofactor of NOS rather than as reactant in L-arginine oxidation. Their conclusions were predominantly based on their findings that exogenously added H₄biopterin was substoichiometrically active although it was not recycled during the reaction (17, 48). They did not consider, however, the possibility that endogenous H₄biopterin tightly bound to the enzyme may be regenerated at the expense of NADPH (17, 19, 28). In support of this latter hypothesis, we found that the stimulatory effects of H₄biopterin were enhanced at increased concentrations of NADPH (see Fig. 4). The effective concentrations of NADPH were similar to those required for the various enzymatic activities of NOS (see Table II), suggesting that binding of NADPH to the enzyme is involved in its interaction with H₄biopterin.
enzyme against inactivation by L-NNA. The catalytic center presence of saturating concentrations of the pteridine. Thus, whereas L-NNA irreversibly inhibits this enzyme does not depend on the presence of calmodulin (47), we speculated that the nitro derivative may act as mechanism-based inhibitor. Indeed, we found that NOS activity was reduced in a time-dependent manner by L-NNA (see Fig. 6). Inactivation of the enzyme caused by preincubation with L-NNA, however, did not depend on the presence of calmodulin (see Table IV), indicating that enzymatic metabolism of the inhibitor does not account for the observed inactivation. It remains unclear, therefore, how L-NNA inactivates NOS. The inhibitor most likely exerts its actions at the substrate-binding site of NOS, since L-arginine was found to protect the enzyme against inactivation by L-NNA. The catalytic center of another P-450 enzyme (P-450mono) was described as being highly sensitive toward steric and electrostatic distortions (51). Accordingly, the voluminous and strongly electron-withdrawing nitro substituent of L-NNA may induce a conformational change at the active site of NOS. Such a profound effect on the catalytic site of brain NOS may also explain why L-NNA inhibits substrate-independent oxygen activation.

Interestingly, N"-nitro derivatives of L-arginine also inhibit substrate-independent activation of oxygen, whereas L-NMA does not (38, 39). Structural similarities between the guanidino residues of L-NNA and NOHLA have been suggested to account for the high affinity of L-NNA to constitutive NOS (50). Since NOHLA serves as a substrate for brain NOS, whereas L-NNA irreversibly inhibits this enzyme (47), we speculated that the nitro derivative may act as mechanism-based inhibitor. Indeed, we found that NOS activity was reduced in a time-dependent manner by L-NNA (see Fig. 6). Inactivation of the enzyme caused by preincubation with L-NNA, however, did not depend on the presence of calmodulin (see Table IV), indicating that enzymatic metabolism of the inhibitor does not account for the observed inactivation. It remains unclear, therefore, how L-NNA inactivates NOS. The inhibitor most likely exerts its actions at the substrate-binding site of NOS, since L-arginine was found to protect the enzyme against inactivation by L-NNA. The catalytic center of another P-450 enzyme (P-450mono) was described as being highly sensitive toward steric and electrostatic distortions (51). Accordingly, the voluminous and strongly electron-withdrawing nitro substituent of L-NNA may induce a conformational change at the active site of NOS. Such a profound effect on the catalytic site of brain NOS may also explain why L-NNA inhibits substrate-independent oxygen activation.

The identification of brain NOS as a P-450-like protein (21, 23) and the data presented in this study suggest a hypothetical reaction scheme of NO biosynthesis as outlined in Fig. 7. One-electron reduction of ferric heme iron, binding of molecular oxygen, and electron transfer from ferrous iron to the oxygen may generate a ferric superoxide species. Subsequently, a second electron enters the reaction, and removal of water results in the formation of a ferryl compound which is generally assumed to be the hydroxylating species in P-450-catalyzed monoxygenase reactions (52, 53). Accordingly, this ferryl species may catalyze the oxidation of bound L-arginine, leading to the formation of NOHLA (depicted as its oxime tautomer). As reported for other P-450 enzymes (54), electron transfer from ferrous iron to enzyme-bound oxygen may result in the release of superoxide anion and thus in the formation of H₂O₂. Even saturating concentrations of L-arginine did not effectively block substrate-independent oxygen activation unless exogenous H₂biopterin was present, indicating that oxygen activation uncouples from substrate oxidation at low reaction rates. The sequence of one-electron transfers leading to the formation of NOHLA, the involvement of a hydroxylating ferryl compound, and uncoupling of oxygen activation from substrate oxidation are in good accordance with well established P-450 chemistry (52-54). The mechanisms that may be involved in the further conversion of NOHLA are less obvious, however, since it seems difficult to reconcile the odd-electron stoichiometry of NO biosynthesis (17, 35) with the
two-electron P-450 chemistry. To overcome this problem it has been speculated that the second electron, which is required for the formation of a hydroxyxylating ferryl species, may be derived from the substrate (20, 21). According to these previous suggestions, NOHLA may be converted into a hypothetical nitrosyl cation radical by donating one electron to ferric heme (see Fig. 7). The equilibrium of this reaction may be shifted toward the formation of the postulated radical by its subsequent cleavage into NO and L-citrulline. The formation of these final reaction products involves binding of oxygen to ferrous heme, one-electron reduction, and subsequent formation of the ferryl species. C-hydroxylation of the nitrosyl cation radical may result in the formation of a hypothetical intermediate which should finally decompose into NO and L-citrulline. The proposed reaction sequence involves two different types of electron transfer, i.e., reduction of ferric heme by NOHLA and reduction of a ferrous heme-oxygen compound by an NADPH-derived electron. These reactions are not supposed to operate on equal redox levels, and in fact, some reductive NOS inhibitors did not only reduce the overall reaction rates of L-citrulline formation, but also induced considerable accumulation of NOHLA. As described in the present study, NOHLA completely blocked NOS-catalyzed generation of NO2, in a turn-over independent manner, indicating that the ferric superoxide species responsible for superoxide release is not formed when the intermediate is bound to the enzyme instead of L-arginine. This may be due to the oxidation of NOHLA to the nitrosyl cation radical (see Fig. 7) because nitrosyl ligands are isoelectronic to CO and known to stabilize the central heme iron in its ferrous state by inducing a charge delocalization from the iron to \( \pi \)-orbitals of the ligand.

Although brain and macrophage NOS exhibit similar cofactor requirements, there appear to be marked differences in L-arginine binding and reduction of oxygen by these isozymes, since the inducible enzyme (i) does not exhibit pronounced substrate-independent NADPH-oxidase activity (35), (ii) is irreversibly inhibited by L-NMA (55), and (iii) is much less sensitive to L-NMA than the brain enzyme (56). A better understanding of these fundamental differences between constitutive and inducible NOS may be crucial for the evaluation of the reaction mechanisms involved in NO synthesis and also for the development of isoform-specific inhibitors.

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**REFERENCES**


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