The Ferrous-dioxy Complex of Neuronal Nitric Oxide Synthase

DIVERGENT EFFECTS OF L-ARGININE AND TETRAHYDROBIOPTERIN ON ITS STABILITY*

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Nitric oxide synthases (NOS) are hemeproteins that catalyze oxidation of L-arginine to nitric oxide (NO) and citrulline. The NOS heme iron is expected to participate in oxygen activation during catalysis, but its interactions with O2 are not characterized. We utilized the heme-containing oxygenase domain of neuronal NOS (nNOSoxy) and stopped-flow methods to study formation and autooxidative decomposition of the nNOSoxy oxygenated complex at 10 °C. Mixing ferrous nNOSoxy with air-saturated buffer generated a transient species oxygenated complex at 10 °C. Mixing ferrous nNOSoxy with air-saturated buffer generated a transient species.

Nitric oxide (NO)1 is an ubiquitous signal molecule involved in the regulation of several activities in the cardiovascular, nervous, and immune systems (1–4). NO is generated by a family of enzymes termed NO synthases (NOSs), which catalyze an NADPH- and O2-dependent two-step oxidation of L-arginine to form NO and citrulline (5, 6). All NOSs are comprised of a C-terminal reductase domain that contains the binding sites for calmodulin (CaM), FMN, FAD, and NADPH and an N-terminal oxygenase domain that contains binding sites for iron protoporphyrin IX (heme), tetrahydrobiopterin (H4B), and substrate (L-arginine) (7–13). A variety of evidence suggests the individual oxygenase and reductase domains can fold and function independently (8–10, 13, 14). The neuronal NOS isoform (nNOS) is constitutively expressed in an inactive form that requires Ca2+-dependent CaM binding to activate its NO synthesis (15, 16). CaM activates NO synthesis in part by triggering an interdomain electron transfer between the flavin and heme centers of nNOS (17, 18).

The NOS heme iron is axially coordinated to the protein via a cysteine thiolate, as is the case for cytochrome P450s, and is predominantly five-coordinate and high spin in its ferric state (19–21). Substrate appears to bind directly above the heme and can interact with ligands bound to the heme iron, such as CO or NO (22, 23). During catalysis of NO synthesis, such positioning may alter heme iron reactivity or sterically specify substrate hydroxylation events as catalyzed by the heme iron. The H4B cofactor is critical for stabilizing NOS dimeric structure and maintaining catalytic activity (24–26), and also affects the heme iron spin state, axial ligand stability, and binding of exogenous ligands (9, 27–29).

Reduction of the NOS heme iron is associated with activation of NO synthesis from L-arginine or with production of superoxide and H2O2 in the absence of substrate (17, 27, 30, 31). Heme iron reduction is thought to be critical for both activities because it may enable the enzyme to bind and activate O2 (5, 6). Indeed, NO synthesis from either L-arginine or the reaction intermediate N6-hydroxylarginine is diminished in the presence of ligands that may prevent O2 binding to the heme iron, such as CO or imidazole (32, 33). Although complexes between the NOS heme iron and CO, NO, and CN− have been characterized spectroscopically (19–23, 34), direct evidence of O2 binding to the NOS ferrous heme iron is not available.

To investigate what role the NOS heme plays in oxygen activation during catalysis, we utilized the oxygenase domain of nNOS2 (nNOSoxy, amino acids 1–720) to study reactions between its ferrous heme iron and O2. This report provides direct evidence for formation of a transient NOS FeO2 complex, and characterizes how bound L-arginine and H4B effect its formation and decay kinetics.

EXPERIMENTAL PROCEDURES

Materials—Oxygen gas was purchased from Liquid Carbonic Company. All other reagents and materials were obtained from Sigma or from sources reported previously (14, 24, 30).

Protein Expression and Purification—Rat nNOS cDNA was a gift from Drs. David Breit and Solomon Snyder at the John Hopkins University. The pCWori plasmid was used to overexpress the nNOSoxy (amino acids 1–720) to study reactions between its ferrous heme iron and O2. This report provides direct evidence for formation of a transient NOS FeO2 complex, and characterizes how bound L-arginine and H4B effect its formation and decay kinetics.
reported by McMillan and Masters (9). PCR was used to amplify the nNOSoxy DNA (corresponding to amino acids 1–720) with an incorporated hexahistidine tag at the C terminus, using as a template a pCIS-2 construct of full-length nNOS. The oligonucleotides used were 5’-ATATTGACCATATGGGAAGAACGACGTTTGGG as the forward primer (to create an Ndel site), and 5’-ATATTGACGACGTTTGGG-GTGGTTGGTTGGTTGGTTGGGCCCTTCCACACG as the backward primer (to create the hexahistidine tag and a stop codon followed by an SalI site). PCR was done using the Expand Long Template system from Boehringer Mannheim. A typical 50-μl mixture included 350 μM dNTPs, 100 pmol of each primer, 10 ng of nNOS template, PCR buffer containing 1.75 mM MgCl₂, and 2.5 units of enzyme mix containing Taq and Pfu DNA polymerases. The PCR product was restricted with Ndel and SalI and ligated into a similarly restricted pCwori plasmid. The product was transformed to competent E. coli BL21(DE3) cells and transformants were selected on LB plates containing 125 mg/L ampicillin. The sequence of the final construct was checked at the DNA core facility of the Cleveland Clinic Foundation.

One liter of Terrific Broth (Life Technologies, Inc.) containing 4 ml of glycerol and 125 mg of ampicillin was inoculated with a 50-ml overnight culture of the bacteria containing the plasmid for expression of nNOSoxy and grown at 25 °C for 2 days. Cells were harvested and suspended in the buffer A (40 mM EPPS, pH 7.6, 10% glycerol, 0.25 mM NaCl) containing protease inhibitors and 1 mg/ml lysozyme, lysed by three cycles of freeze-thawing, and sonicated (three times at 30 s each with a 1-min rest on ice between the pulses). Cell-free supernatant obtained after centrifugation at 30,000 g for 50 min at 4 °C was applied to a column containing Ni²⁺-nitrilotriacetic acid resin (10 ml) equilibrated with buffer A containing 1 mM PMSF and then eluted with buffer A containing 40 mM imidazole and 1 mM PMSF, and 1.5-ml fractions were collected. Fractions containing nNOSoxy were pooled and concentrated using a Centriprep-30. The protein was dialyzed against buffer A with 1 mM dithiothreitol and stored in aliquots at −70 °C. The purity of the protein was estimated to be 90% as judged by SDS-polyacrylamide gel electrophoresis. The hemeprotein concentration was estimated based on the absorbance at 444 nm for the ferrous-CO complex, using an extinction coefficient of 76 mM−1 cm−1 (21).

Sample and Solution Preparation—The nNOSoxy samples were dialyzed overnight at 4 °C against 40 mM Bis-Tris propane, pH 7.4, containing 1 mM dithiothreitol alone or plus 4 mM H₂B, plus 3 mM L-arginine, or plus H₂B and L-arginine. L-Arginine and H₂B saturation of nNOSoxy samples was confirmed spectroscopically (35). The protein solutions were made anaerobic by repeated cycles of evacuation and equilibration with catalyst-deoxygenated N₂ before use in stopped-flow experiments.

Solutions of 40 mM Bis-Tris propane, pH 7.4, containing various concentrations of O₂ were prepared by mixing different volumes of O₂-saturated buffer with air-saturated or anaerobic buffer solutions. Saturation was achieved by bubbling O₂ for 2 h in a septum-sealed flask at 21 °C. Final O₂ concentrations were calculated based on a saturating O₂ concentration of 1.2 mM at 21 °C.

Optical Spectroscopy—Anaerobic spectra of ferrous nNOSoxy were recorded at 15 °C in septum-sealed quartz cuvettes that could be attached through a ground-glass joint to a vacuum gas train.

Rapid Kinetic Measurements—Measurements were obtained using a stopped-flow apparatus from Hi-tech Ltd. (model SF-51) equipped for anaerobic work. Measurements were carried out at 10 °C and initiated by mixing anaerobic solutions of 5 μM nNOSoxy that had been pre-reduced with excess dithionite (40 μM) with an equal volume of buffer containing O₂ at different concentrations. Formation and decay of the oxygenated nNOSoxy complex were monitored at 410 nm, 405 nm, or at other wavelengths as indicated in the text. In some experiments, the stopped-flow instrument was equipped with a rapid-scanning detector (Hi-Tech, MG-3000) designed to collect a complete spectrum (200–800 nm) within 90 ms. The detector was calibrated relative to four principal peaks: Soret absorption wavelengths of a dicyanomethylene filter (BG 290) and a 550-nm filter.
lowed by a slower increase in absorbance. When the reaction was monitored at 440 nm (lower panel), the direction of absorbance change was reversed but otherwise proceeded with identical kinetics. At either wavelength, the absorbance observed at the start of the reaction can be attributed to ferrous nNOSoxy, while the absorbance at the inflection and end points can be attributed to sequential formation of the transient intermediate and ferric nNOSoxy, respectively. We thus monitored the reaction at a range of single wavelengths and plotted the maximum absorbance obtained for each of the three species as a function of wavelength. As shown in Fig. 3, the spectra so derived match the rapid scanning spectra reported in Fig. 1 and are consistent with the sequential nature of the proposed reaction. Fitting each stopped-flow trace obtained at single wavelengths to a two-exponential function showed that there was no variation in the observed rate constants for formation or autooxidation. This indicates that the transient intermediate and ferric nNOSoxy are the only two observable products of the reaction.

We next examined the formation and decay of the transient species as a function of O₂ concentration by monitoring absorbance change at 410 nm. The pseudo-first order rate constants obtained for its formation in the presence of saturating L-arginine and H₄B are plotted as a function of O₂ concentration in panel A of Fig. 4. The association and dissociation rate constants derived from the graph are listed in Table I along with rate constants obtained under similar conditions for nNOSoxy samples saturated either with L-arginine or H₄B alone or in the absence of both molecules.

As shown in panel B of Fig. 4, the decay rate of the transient species did not change with O₂ concentration, indicating decay is independent of dissolved O₂. The autooxidation rate for the H₄B- and L-arginine-saturated protein was best fit to a single exponential function and was therefore monophasic, giving a rate of 10 s⁻¹ (Table I). An identical monophasic decay rate was also observed with nNOSoxy that was saturated with H₄B alone (Table I). However, decay of the transient species in nNOSoxy samples devoid of both L-arginine and H₄B was bi-phasic, giving rate constants of 2.3 s⁻¹ and 0.12 s⁻¹ (Table I). Again, these decay rates did not change as a function of O₂ concentration (data not shown). With an nNOS sample satu-
rates with l-arginine alone, only the slow decay rate was observed (Table I).

**DISCUSSION**

These data provide the first direct evidence for O₂ binding to the NOS heme iron, consistent with its proposed role in oxygen activation during NO synthesis (5, 6). Mixing an O₂-containing solution with ferrous nNOSoxy resulted in rapid formation of a transient intermediate whose spectral and kinetic characteristics were quite similar to the Fe(II)O₂ complexes of a number of cytochrome P-450s (36–44), identifying the intermediate as Fe(II)O₂ nNOSoxy. Although its Soret maxima (427 nm) is somewhat red-shifted compared with most Fe(II)O₂ cytochrome P-450s which absorb maximally at 418–420 nm (36–40, 42–44), it is most similar to the Fe(II)O₂ complex of substrate-bound cytochrome P-450s which absorbs maximally at 423 nm (41). The spectral features of Fe(II)O₂ nNOSoxy did not change noticeably in the absence of bound substrate (l-arginine) or H₂B (data not shown), consistent with bound substrate also not altering the visible spectra of the ferrous-CO or -NO complexes of nNOS (19, 23, 30).

In air-saturated solution, formation of the nNOSoxy Fe(II)O₂ complex occurred at rates that were 40–4000 times faster than complex decay. Under such circumstances, practically all of the nNOSoxy sample exists in its Fe(II)O₂ form prior to decay.³ Rates of complex formation and decay were invariant as a function of wavelength and both processes generated spectral isosbestic points. This indicates that complex formation and decay occur without formation of other observable intermediates, which is also the case for most cytochrome P450s examined to date (36, 40). Formation of Fe(II)O₂ nNOSoxy was first order with respect to O₂, reversible, and followed a simple one-step mechanism. Decay of the complex was independent of O₂ concentration, occurred via a one- or two-exponential process depending on sample conditions, and generated ferric nNOSoxy as a product. A model consistent with the data is shown in Scheme 1. Mechanisms proposed for the autooxidation of Fe(II)O₂ heme proteins generally involve electron transfer to O₂ as a primary step to form superoxide, which undergoes further irreversible reactions (45, 46). Indeed, uncoupled NADPH oxidation by nNOS is reported to generate superoxide as a primary product (31). However, because cytochrome P-450s can also generate H₂O₂ or water as primary products of uncoupled oxidation (36, 47, 48), it is possible that nNOS may form these products under favorable conditions.

³ This enabled us to estimate an extinction coefficient for the nNOSoxy Fe(II)O₂ complex (64.1 mm⁻¹ cm⁻¹ at 427 nm), which is lower than the estimated extinction coefficients of ferric or ferrous nNOSoxy (76 and 69.1 mm⁻¹ cm⁻¹ at 400 and 414 nm, respectively).

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**TABLE I**

*Rate constants for formation and decay of the oxygenated nNOSoxy complex*

| Conditions               | kₚ | kₑ   | Decay |
|-------------------------|----|------|-------|
| l-Arginine − H₂B        | 1.4 × 10⁶ | 177 | 0.12, 2.3 |
| +l-Arginine − H₂B       | 1.1 × 10⁶ | 65 | 0.14   |
| −l-Arginine + H₂B       | 7.8 × 10⁵ | 130 | 10     |
| +l-Arginine + H₂B       | 9.0 × 10⁵ | 108 | 10     |

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**Scheme 1**

\[
\begin{align*}
\text{Heme Fe}^{III} + O_2 & \rightarrow \text{Heme Fe}^{IV}O_2 \\
\text{Heme Fe}^{IV}O_2 & \rightarrow \text{Heme Fe}^{V}O_2 + O_2 \\
\text{Heme Fe}^{V}O_2 & \rightarrow \text{Heme Fe}^{VI}O_2 + O_2
\end{align*}
\]

**Scheme 2**

\[
\begin{align*}
\text{Heme Fe}^{III} & \rightarrow \text{Heme Fe}^{IV}O_2 \\
\text{Heme Fe}^{IV}O_2 & \rightarrow \text{Heme Fe}^{V}O_2 + O_2 \\
\text{Heme Fe}^{V}O_2 & \rightarrow \text{Heme Fe}^{VI}O_2 + O_2
\end{align*}
\]
steps of NO synthesis may require that a second electron be provided to the Fe$^{4+}$O$_2$ complex. When viewed this way, destabilization of the Fe$^{3+}$O$_2$ complex by H$_4$B seems counterproductive, because it would favor a decomposition reaction that yields ferric NOS and superoxide instead of further reductive steps that lead to NO synthesis. However, the fact that NO synthesis by nNOS is tightly coupled to NADPH oxidation in the presence of saturating H$_4$B (5, 6) suggests that decay of the Fe$^{3+}$O$_2$ species under normal reaction conditions is slower than the additional reductive steps required to activate oxygen for productive catalysis.

Recent work with H$_2$B-free forms of nNOS (27) and inducible NOS$^*$ shows that they can catalyze NADPH-dependent, heme iron-based O$_2$ consumption in the presence of bound l-arginine without catalyzing NO synthesis. This implies that formation of oxygenated iron species within the active site can occur in the absence of H$_2$B but is itself not sufficient to support NO synthesis. Thus, we speculate that H$_2$B destabilization of the nNOSoxy Fe$^{3+}$O$_2$ complex as described here may actually reflect a more global influence of H$_2$B on the reactivity of all nNOS iron-oxo species that somehow enables oxygen activation at the heme to become coupled to NO synthesis. Our current findings provide a foundation to explore this hypothesis.

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