Substrate-specific Interactions with the Heme-bound Oxygen Molecule of Nitric-oxide Synthase*

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We report the characterization by resonance Raman spectroscopy of the oxygenated complex (FeIV=O) of nitric-oxide synthases of Staphylococcus aureus (saNOS) and Bacillus subtilis (bsNOS) saturated with N⁶-hydroxy-L-arginine. The frequencies of the νFe-O and νO-O modes were 530 and 1135 cm⁻¹, respectively, in both the presence and absence of tetrahydrobiopterin. On the basis of a comparison of these frequencies with those of saNOS and bsNOS saturated with L-arginine (νFe-O at 517 cm⁻¹ and νO-O at 1123 cm⁻¹) and those of substrate-free saNOS (νFe-O at 517 and νO-O at 1135 cm⁻¹) (Chartier, F. J. M., Blais, S. P., and Couture, M. (2006) J. Biol. Chem. 281, 9953–9962), we propose two models that account for the frequency shift of νFe-O (but not νO-O) upon N⁶-hydroxy-L-arginine binding as well as the frequency shift of νO-O (but not νFe-O) upon L-arginine binding. The implications of these substrate-specific interactions with respect to catalysis by NOSs are discussed.

Nitric-oxide synthases (NOSs)² are a family of heme proteins that synthesize NO by catalyzing the two-step oxidation of L-arginine using an O₂-dependent mechanism (1–5). NOSs first hydroxylate L-arginine to N⁶-hydroxy-L-arginine (NOHA). This intermediate then becomes a substrate for the second reaction, which produces 1-citrulline and NO.

To carry out these two reactions, the heme iron at the active site of NOs must first be reduced to be able to bind molecular oxygen (O₂) and to form an oxygenated complex (FeIV=O). The electron is supplied by NADPH via the reductase domain in mammalian NOSs and from an independent reductase for the bacterial NOSs (6). The FeIV=O complex must then be activated to form the oxidizing intermediate that oxidizes the substrates (L-arginine and NOHA). The hydroxylation of L-arginine requires an additional external electron initially supplied by tetrahydrobiopterin (H₄B) to allow oxygen activation. Oxygen activation is thought to occur via a mechanism similar to that proposed for cytochrome P450 and to involve a Compound I intermediate defined as an oxyferryl (FeIV=O) heme with an associated radical (2, 5, 7–9). Strong evidence suggests that an electron and possibly a proton are supplied from the H₄B cofactor to form a peroxo (FeIVO₅²⁻) or hydroperoxy (FeIVOO⁻H) complex (10). A second proton is then recruited, leading to the heterolytic cleavage of the O–O bond and the formation of a water molecule and a Compound I-type intermediate.

Unlike the hydroxylation of L-arginine, the conversion of NOHA to NO and citrulline requires only a transiently supplied electron to activate the heme-bound O₂. Many mechanisms for the hydroxylation of NOHA have been put forward (8, 11–13). In general, it has been suggested that an activated form of the oxygenated complex (peroxo or hydroperoxy), produced after the transfer of an electron from H₄B, can perform an electrophilic attack on the guanidino carbon of NOHA to form a tetrahedral intermediate that undergoes conversion to 1-citrulline and NO with a concomitant electron donation back to H₄B (14).

NOSs likely evolved two different mechanisms of oxygen activation to oxidize their two substrates in the first and second catalytic cycles, respectively. Interactions between the oxygen atoms of the FeIV=O complex and the substrates, together with the electron/proton donor properties of the cofactor, likely play critical roles in determining how NOSs catalyze these reactions. Although H₄B has been more extensively characterized, including its involvement as an electron donor in the first and second reactions (10, 14–18) as well as its likely role as a proton donor (19, 20), specific details on the interactions between the heme-bound O₂ and substrates and the involvement of H₄B in modulating these interactions are scarce (21–23).

We used stopped-flow spectrophotometry and continuous-flow resonance Raman spectroscopy to probe the FeIV=O complex of Staphylococcus aureus NOS (saNOS) with the substrate L-arginine (23). Our results showed that L-arginine binding causes a downshift in the frequency of the νO-O mode but has no effect on the frequency of the νFe-O mode (23). A similar downshift of the νO-O frequency has also been reported for the oxygenated complex of mammalian neuronal NOS (nNOS) (24). Analysis of the kinetics of formation and decay of the FeIV=O complex revealed that L-arginine stabilizes the complex against autoxidation to the ferric form in saNOS. These findings indicate that hydrogen-bonding interactions involving the heme-bound O₂ and L-arginine stabilize the FeIV=O complex.

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2 The abbreviations used are: NOSs, nitric-oxide synthases; NOHA, N⁶-hydroxy-L-arginine; H₄B, tetrahydrobiopterin; saNOS, S. aureus nitric-oxide synthase; nNOS, mammalian neuronal nitric-oxide synthase; bsNOS, B. subtilis nitric-oxide synthase; INOS, mammalian inducible nitric-oxide synthase; ENOS, mammalian endothelial NOS.
against autoxidation. Hydrogen-bonding interactions also decrease the frequency of the $v_{\text{O-O}}$ mode by modulating the amount of $\pi$-backboning from the heme iron (23).

To complement our first study of the Fe$^{6+}$O$_2$ complex of saNOS in the presence of L-arginine (23), we now report an investigation of the interactions of the Fe$^{6+}$O$_2$ complex in the presence of NOHA for saNOS and Bacillus subtilis NOS (bsNOS) by resonance Raman spectroscopy. Specifically, we report the frequencies of the $v_{\text{O-O}}$ and $v_{\text{Fe-O}}$ modes of NOHA-bound saNOS and bsNOS as well as of L-arginine-bound bsNOS. Differences in the frequencies of the $v_{\text{Fe-O}}$ and $v_{\text{O-O}}$ modes were observed in the presence of NOHA with respect to those measured with L-arginine, indicating that the heme-bound O$_2$ is involved in substrate-specific interactions in both NOSs. The implications of these results for the mechanisms of oxygen activation by NOSs are discussed.

EXPERIMENTAL PROCEDURES

Materials—H$_2$B and NOHA were purchased from Sigma. Argon and $^{16}$O$_2$ gas were from Praxair, Inc. (Mississauga, ON, Canada). $^{18}$O$_2$ gas (99% purity) was from ICON (Mt. Marion, NY).

Enzyme Preparation—saNOS and bsNOS were expressed in Escherichia coli from the cloned genes and purified as described previously (25, 26). Samples were maintained in 40 mM HEPES (pH 7.6), 150 mM NaCl, and 1 mM DL-dithiothreitol. Where indicated, NOHA (500 $\mu$M) was added to the purified enzymes. For samples containing H$_2$B, a concentration of 500 $\mu$M was used. The association of H$_2$B and NOHA with saNOS and bsNOS and that of L-arginine with bsNOS were monitored by measuring the displacement of DL-dithiothreitol bound to the ferric enzymes by optical spectroscopy (25).

Stopped-flow Spectroscopy—Stopped-flow experiments were carried out as described previously (23). Briefly, anaerobic protein samples (5 $\mu$L) were reduced with the minimum amount of sodium dithionite required to reduce the heme. Complete reduction of the sample was verified by optical spectroscopy. Rapid mixing experiments with reduced saNOS and bsNOS and molecular oxygen were carried out in 21% O$_2$ gas (100%) was used to saturate anaerobic buffer with O$_2$. This solution was then used to prepare buffers of the specified oxygen concentrations.

The kinetics of formation and decay of the Fe$^{6+}$O$_2$ complex were followed at individual wavelengths in kinetic scanning mode. Kinetic traces were recorded at 5-nm intervals from 380 to 460 nm and from 515 to 610 nm, which generated optical spectra versus time data sets. The kinetic data were analyzed using SPECFIT global analysis software (Spectrum Software Associates, Chapel Hill, NC) with a kinetic model involving four states for the data obtained from saNOS and bsNOS saturated with NOHA: state A corresponded to the initial reduced protein; state B corresponded to the transient Fe$^{6+}$O$_2$ complex; state C corresponded to an intermediate; and state D corresponded to the resting ferric form. For the data obtained from bsNOS saturated with L-arginine, a three-state model was used: state A corresponded to the initial reduced protein; state B corresponded to the transient Fe$^{6+}$O$_2$ complex; and state C corresponded to the resting ferric form. The deconvoluted optical spectra, the fits at all wavelengths, and the time course of the appearance and decay of the four kinetic states were obtained from these analyses.

Resonance Raman Spectroscopy—Resonance Raman spectra of the oxygenated intermediates were acquired with a custom-made continuous-flow T-mixer as described previously (23). Prior to mixing, the reduced forms of saNOS and bsNOS (80 $\mu$M) were prepared by equilibrating the enzymes with pure argon gas for at least 30 min at room temperature and by adding the minimum amount of sodium dithionite required to reduce the heme completely. Optical spectra in the visible region (from 450 to 700 nm), recorded directly from the 10-ml syringe containing the reduced proteins, were recorded to assess the complete reduction of the protein. Pure $^{16}$O$_2$ and $^{18}$O$_2$ gases were used to prepare oxygenated buffers of known concentrations. The rapid mixer was made anaerobic using a 10-mx sodium dithionite solution, followed by washing with anaerobic buffer to remove the dithionite. Oxygenated buffers ($^{16}$O$_2$ and $^{18}$O$_2$ in two separate mixing experiments) and the reduced proteins were mixed at a 1:1 ratio from 10-ml syringes. The output at 441.6 nm from a helium-cadmium laser (Liconix laser, Melles Griot, Ottawa, Ontario) at $\sim$10 milliwatts was focused on the sample inside the channel of the quartz flow cell. The resonance Raman spectra of saNOS/NOHA/H$_2$B and saNOS/NOHA samples were acquired 4.5 and 10 ms after mixing, respectively, using previously described equipment (25). Those of bsNOS/L-Arg and bsNOS/NOHA were acquired 22 and 32 ms after mixing, respectively. Several 30-s spectra (12–15) were acquired and averaged. All resonance Raman spectra were obtained at room temperature (25°C) and were calibrated with the lines of indene. The spectrum of reduced myoglobin was recorded before each experiment to check for small calibration differences on different days. The spectrum of the buffer was also recorded for each experiment to subtract the quartz diffraction signal originating from the detection tube of the continuous-flow mixer.

RESULTS

Stopped-flow Spectroscopy—Stopped-flow optical spectroscopy was used to obtain the rates of formation and decay of the Fe$^{6+}$O$_2$ complex of saNOS in the presence of NOHA (without H$_2$B) (Fig. 1). The kinetics of formation and decay of the Fe$^{6+}$O$_2$ complex of saNOS in the presence of NOHA and H$_2$B have already been reported (Table 1) (23). Reduced saNOS saturated with NOHA was mixed with O$_2$-saturated buffer. The data were acquired by kinetic scanning in the 380–460 nm (Soret region) (Fig. 1A) and 515–610 nm (supplemental Fig. 1) regions of the absorption spectrum. These kinetic scanning data were first studied by global analysis using a three-state sequential kinetic model (A $\rightarrow$ B $\rightarrow$ C). The three-state model did not fit the data properly (supplemental Fig. 2), and inconsistent rates were calculated from the two optical region data sets.

A four-state model (A $\rightarrow$ B $\rightarrow$ C $\rightarrow$ D) best fit the data and produced consistent results in both regions of the absorption spectrum (Fig. 1 and supplemental Fig. 1). The kinetic traces recorded at 430 and 450 nm and the corresponding fits to the four-state model are shown in Fig. 1B. These wavelengths show that four states are indeed needed to fit all the transitions
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FIGURE 1. Kinetics of formation and decay of the Fe\(_{\text{II}}\)O\(_{2}\) complex of saNOS in the presence of NOHA. A, raw optical spectra in the Soret region. The spectra acquired at 1.5 ms (solid line), 11 ms (dashed line), 71 ms (dotted line), and 588 ms (dotted/dashed line) after the initiation of the reactions are identified. B, variations in the absorbance at 430 nm (circles) and 450 nm (triangles) and fits to the four-state kinetic model (solid lines). C, residuals of the fits at all wavelengths as a function of time. All the residuals showed \(\lambda_4 < 0.0008\) and were randomly distributed, indicating a good fit of the data to the four-state kinetic model. D, variations in the concentrations of the four states as a function of time. The solid line is reduced saNOS; the dashed line is the Fe\(_{\text{II}}\)O\(_{2}\) complex; the dotted line is state C; and the dashed/dotted line is the resting ferric form. E, calculated spectra in the Soret region of the reduced form (solid line), Fe\(_{\text{II}}\)O\(_{2}\) (dashed line), state C (dotted line), and the ferric form (dotted/dashed line). F, calculated spectra in the visible region of reduced saNOS (solid line), the Fe\(_{\text{II}}\)O\(_{2}\) complex (dashed line), state C (dotted line), and the ferric form (dotted/dashed line). Detailed analysis of the data in the visible region is shown in supplemental Fig. 1. The NOHA concentration was 500 \(\mu\)M.

observed. The residuals of the fits at all wavelengths were small in amplitude and randomly distributed (Fig. 1C), indicating that a good fit to the four-state model was obtained. State A, with a Soret optical transition centered at 410 nm, corresponded to reduced saNOS (Fig. 1E, solid line) and reacted with molecular oxygen at a rate of 230 s\(^{-1}\) to form the Fe\(_{\text{II}}\)O\(_{2}\) complex (state B), which had a Soret absorption maximum centered at 425 nm (Fig. 1E, dashed line; and Table 1). State B was identified as an Fe\(_{\text{II}}\)O\(_{2}\) complex from the isotope shifts induced by \(^{16}\)O\(_{2}\) and \(^{18}\)O\(_{2}\) in the resonance Raman spectra (see below). The Fe\(_{\text{II}}\)O\(_{2}\) complex subsequently decayed at a rate of 34 s\(^{-1}\) to an intermediate (state C), which had a broad Soret band (Fig. 1E, dotted line). This intermediate may be composed of a mixture of several states. Finally, the enzyme returned at a rate of 1.6 s\(^{-1}\) to the resting ferric state, which had a Soret optical transition at 400 nm (Fig. 1E, dotted/dashed line; and Table 1). In the 515–610 nm region (Fig. 1F), reduced saNOS had a wavelength absorption maximum centered at 552 nm (solid line); the Fe\(_{\text{II}}\)O\(_{2}\) complex had two maxima near 556 and 570 nm (dashed line); state C had one maximum at 554 nm (dotted line); and the ferric form, which is five-coordinate and high spin, did not have any strong absorption maximum in this optical region (dotted/dashed line). Notably, the absorption bands at 550 and 580 nm observed in the stopped-flow mixing experiments of O₂ with reduced saNOS saturated with NOHA and H\(_{4}\)B (23), which are characteristic of the Fe\(_{\text{III}}\)NO complex, were not observed here. Previous studies have shown that an Fe\(_{\text{III}}\)NO complex is not formed in single turnover experiments from NOHA with pterin-free NOs (27–29), thus supporting the crucial role for H\(_{4}\)B in the synthesis of NO from NOHA. Our studies are no exception. We detected NO synthesis as an Fe\(_{\text{III}}\)NO complex in single turnover experiments with saNOS/NOHA/H\(_{4}\)B (23), but not with NOHA alone (Fig. 1). Therefore, even though the rate of decay of the oxygenated complexes of saNOS/NOHA is only moderately increased in the presence of H\(_{4}\)B (Table 1), the presence of H\(_{4}\)B is nevertheless necessary to observe the Fe\(_{\text{III}}\)NO complex in the course of the reactions of saNOS/NOHA with O₂, thus implying that it is necessary for NO synthesis.

Resonance Raman Spectroscopy in the High Frequency Region of the Fe\(_{\text{II}}\)O\(_{2}\) Complex—To characterize the oxygenated complexes of saNOS with NOHA and H\(_{4}\)B and with NOHA alone, we used a custom-made continuous-flow mixer with a dead time of 0.5 ms (23). The high frequency region of the resonance Raman spectra was first obtained to determine the oxidation and spin states of the Fe\(_{\text{II}}\)O\(_{2}\) complex. The spectra were recorded at 4.5 and 10 ms after mixing reduced saNOS/NOHA with the O₂-saturated buffer in the presence and absence of H\(_{4}\)B, respectively. At these times, the Fe\(_{\text{II}}\)O\(_{2}\) complexes had reached maximum concentrations of 81 and 72% of the heme available for saNOS/NOHA with (23) and without H\(_{4}\)B (Fig. 1D), respectively. The spectra showed that the oxidation state marker band \(\nu_3\) was at 1374 cm\(^{-1}\) and that the coordination and spin state marker bands \(\nu_3\) and \(\nu_4\) were at 1501 cm\(^{-1}\) in samples with H\(_{4}\)B (Fig. 2, traces A and B) and without H\(_{4}\)B (traces C and D). The Fe\(_{\text{II}}\)O\(_{2}\) complexes of saNOS/NOHA and saNOS/NOHA/H\(_{4}\)B were thus ferric and low spin like the oxygenated complexes of other heme proteins (24, 30).

The shoulder on the \(\nu_3\) mode at 1349 cm\(^{-1}\) and the small intensity at 1468 cm\(^{-1}\) indicated that a small amount of reduced five-coordinate saNOS was present, likely the remains of starting material predicted to correspond to 12 and 11% of the reaction mixtures at 4.5 and 10 ms following the initiation of the reactions based on the kinetic data with NOHA/H\(_{4}\)B (23) and NOHA (Fig. 1D), respectively. These assignments are supported by the resonance Raman spectra of
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TABLE 1
Rates of formation and decay of the oxygenated complexes of saNOS at 21 °C and the wavelength maxima of the Soret absorption bands of the oxygenated complexes

| Protein | Rate of Fe³⁺O₂ formation (s⁻¹) | Rate of decay (fraction) | Soret (nm) | Ref. |
|---------|-------------------------------|--------------------------|------------|-----|
| saNOS   | 1880                          | 39.6 (100%)              | 430        | 23  |
| saNOS/ω-Arg | 106                          | 5.6 (14%)                | 430        | 23  |
| saNOS/H₄B | 2080                         | 32.8 (78%)               | 425        | 23  |
| saNOS/ω-Arg/H₄B | 143                  | 18.0 (67%)               | 405        | 23  |
| saNOS/NOHA | 230                         | 34 (100%)                | 425        | This work |
| saNOS/NOHA/H₄B | 454                    | 22.2 (100%)              | 428        | 23  |
| bsNOS/ω-Arg | 201                         | 1.6 (100%)               | 430        | This work |
| bsNOS/ω-Arg | 60                          | 0.4 (100%)               | 427        | 28  |
| bsNOS/NOHA,d | 94                          | 4.9 (100%)               | –421       | This work |

* The rate of decay corresponds to autoxidation when catalysis cannot occur, i.e., with either the substrate or H₄B absent. The rate of decay corresponds to catalysis when ω-arginine or NOHA is present along with H₄B.
* The experiments were performed using buffer saturated with 10% O₂.
* The oxygenated intermediate with the Soret absorption band at 430 nm was converted to the oxygenated intermediate with a Soret absorption band at 430 nm at a rate of 89 s⁻¹.
* The experiment was performed using buffer saturated with 100% O₂. The rate of conversion of state C to the ferric form was 1.6 s⁻¹ for saNOS and 1.2 s⁻¹ for bsNOS.
* The experiment was performed using buffer saturated with 100% O₂. The rate of conversion of the FeIIINO complex to the ferric form was 3.8 s⁻¹.
* The experiments were performed using buffer saturated with 10% O₂.
* The rate of decay corresponds to autoxidation when catalysis cannot occur, i.e., with either the substrate or H₄B absent. The rate of decay corresponds to catalysis when ω-arginine or NOHA is present along with H₄B.

The rate of decay corresponds to autoxidation when catalysis cannot occur, i.e., with either the substrate or H₄B absent. The rate of decay corresponds to catalysis when ω-arginine or NOHA is present along with H₄B.

FIGURE 2. High frequency region of the resonance Raman spectra of the Fe³⁺O₂ complexes of saNOS with NOHA/H₄B and with NOHA alone. The resonance Raman spectra were recorded 4.5 ms after mixing reduced saNOS/NOHA/H₄B with ¹⁶O₂-saturated (trace A) and ¹⁸O₂-saturated (trace B) buffers and 10 ms after mixing saNOS/NOHA with ¹⁶O₂-saturated (trace C) and ¹⁸O₂-saturated (trace D) buffers. The ¹⁶O₂-minus-¹⁸O₂ difference spectra of the samples containing NOHA/H₄B (trace E, trace A minus trace B) and NOHA (trace F, trace C minus trace D) are shown. The ¹⁶O₂-minus-¹⁸O₂ difference spectrum of saNOS/NOHA recorded 81 ms after mixing is shown in trace G. The excitation wavelength was 441.6 nm, and the power was 12 milliwatts. The traces marked with asterisks correspond to lines from the laser.

saNOS/NOHA obtained later after the initiation of the reactions (Fig. 3). At 56 ms (Fig. 3, trace B) and 81 ms (trace A) after mixing, the ν₃ and ν₄ lines of reduced saNOS were no longer observed, unlike the 10 ms spectrum (trace C). These results are consistent with stopped-flow data indicating that no reduced form remained at those times (Fig. 1D). Also consistent with stopped-flow data, the spectrum obtained at 81 ms displayed a ν₃ line at 1488 cm⁻¹, which corresponded to ferric five-coordinate saNOS and which represented 8% of the heme at that time. Also, at 81 ms, state C had reached its maximum concentration. Our results show that, with a ν₃ line at 1372 cm⁻¹ and a ν₄ line at 1501 cm⁻¹, this intermediate was mostly six-coordinate and low spin.

Isotopic substitution was used to identify an oxygen-sensitive mode in the high frequency region. The ¹⁶O₂-minus-¹⁸O₂ difference spectra of saNOS/NOHA/H₄B (Fig. 2, trace E) and saNOS/NOHA (trace F) revealed the presence of single isotope-sensitive lines at 1135 cm⁻¹ with ¹⁶O₂ and 1070 cm⁻¹ with ¹⁸O₂. These lines corresponded to the νₒ-Oₚ mode, as the isotopic shift of 65 cm⁻¹ was on par with the value expected for an O=O diatomic harmonic oscillator. The frequency was the same as that of substrate-free saNOS (Table 2). These results demonstrate that NOHA and H₄B did not change the frequency of the νₒ-Oₚ mode with respect to that of the Fe⁴⁺O₂ complex of substrate-free and pterin-free saNOS at 1135 cm⁻¹ (Table 2). However, the line at 1135 cm⁻¹ was significantly sharper in the spectrum of the Fe⁴⁺O₂ complex with NOHA (width at a half-height of 20 cm⁻¹) than in that of substrate-free saNOS (width at a half-height of 22 cm⁻¹). In the ¹⁶O₂-minus-¹⁸O₂ difference spectrum of saNOS/NOHA recorded 81 ms after mixing (Fig. 2, trace G), the intensity of the νₒ-Oₚ line was much lower than at 10 ms (trace F), indicating that only a small amount of the Fe⁴⁺O₂ complex remained after 81 ms, which was expected based on the stopped-flow data (Fig. 1D).
TABLE 2

Frequencies of the $v_{\text{Fe-O}}$ and $v_{\text{O-O}}$ modes of saNOS, nNOS, iNOS, and P450$_{\text{cam}}$

For the $v_{\text{Fe-O}}$ modes of saNOS, bsNOS, and the nNOS (nNOSox), and iNOS (iNOSox) oxygenase domains, the predominant mode is indicated in boldface. ND, not detected.

| Protein                  | $v_{\text{Fe-O}}$ ($^{16}\text{O}_2$) cm$^{-1}$ | $v_{\text{O-O}}$ ($^{18}\text{O}_2$) cm$^{-1}$ | Ref. |
|--------------------------|-----------------------------------------------|-----------------------------------------------|------|
| saNOS                    | 517 (487)                                     | 1135 (1071)                                   | 23   |
| saNOS/Arg ($\pm \text{His}$) | 517 (487)                                     | 1123 (1062), 1135 (1071)                      | 23   |
| saNOS/H$_4$B             | 517 (487)                                     | 1123 (1062), 1135 (1071)                      | 23   |
| saNOS/NOHA ($\pm \text{His}$) | 530 (497)                                     | 1135 (1070)                                   | This work |
| bsNOS/Arg                | 530 (497)                                     | 1124 (1064), 1136 (1073)                      | This work |
| bsNOS/NOHA               | 530 (497)                                     | 1137 (1071)                                   | This work |
| nNOSox                   | ND                                            | 1135 (1068)                                   | 22, 24 |
| nNOSox/Arg ($\pm \text{His}$) | ND                                            | 1135 (1068), ~1122 (~1056)                    | 22, 24 |
| nNOSox/H$_4$B            | ND                                            | 1135 (1068)                                   | 22, 24 |
| nNOSox/NOHA ($\pm \text{His}$) | ND                                            | 1135 (1068)                                   | 22, 24 |
| iNOSox                   | ND                                            | 1133 (1067)                                   | 35   |
| iNOSox/Arg ($\pm \text{His}$) | ND                                            | 1126 (1060)                                   | 35   |
| iNOSox/NOHA ($\pm \text{His}$) | ND                                            | 1132 (1066)                                   | 35   |
| P450$_{\text{cam}}$/camphor | 541 (511)                                     | 1140 (1074)                                   | 60, 63 |
| P450$_{\text{cam}}$/adamantanone | 537 (510)                                     | 1147 (1080)                                   | 60   |
| D251N P450$_{\text{cm}}$ | 536 (505)                                     | 1136 (1070)                                   | 30   |
| D251N P450$_{\text{cm}}$/putidaredoxin | 521 (487), 536 (505) | 1129 (1062), 1137 (1071) | 30   |

FIGURE 4. Low frequency region of the resonance Raman spectra of the Fe$^{II}$O$_2$ complexes of saNOS/NOHA and saNOS/NOHA/H$_4$B. The resonance Raman spectra were recorded 4.5 ms after mixing reduced saNOS/NOHA/H$_4$B with $^{16}$O$_2$-saturated (trace A) and $^{18}$O$_2$-saturated (trace B) buffers, 10 ms after mixing reduced saNOS/NOHA with $^{16}$O$_2$-saturated (trace C) and $^{18}$O$_2$-saturated (trace D) buffers, and 81 ms after mixing reduced saNOS/NOHA with $^{16}$O$_2$-saturated (trace E) and $^{18}$O$_2$-saturated (trace F) buffers. The $^{16}$O$_2$-minus-$^{18}$O$_2$ difference spectra of the samples containing NOHA/H$_4$B (trace G) and NOHA alone recorded 10 ms (trace H) and 81 ms (trace I) after mixing are shown. The excitation wavelength was 441.6 nm, and the power was 12 milliwatts.

quency region to identify the $v_{\text{Fe-O}}$ mode. The spectra shown in Fig. 4 were acquired at the same time after mixing as those in Fig. 2. The $^{16}$O$_2$-minus-$^{18}$O$_2$ difference spectra of saNOS/NOHA/H$_4$B (Fig. 4, trace G) and saNOS/NOHA (trace H) revealed the presence of isotope-sensitive lines centered at 530 cm$^{-1}$ with $^{16}$O$_2$ and 497 cm$^{-1}$ with $^{18}$O$_2$. These low intensity lines were observed in several independent mixing experiments (two for saNOS/NOHA and three for saNOS/NOHA/H$_4$B). In all cases, the same isotopic shift was observed. Based on the 33 cm$^{-1}$ isotopic shift and the frequency, which was similar to that of the $v_{\text{Fe-O}}$ mode of other heme proteins (Table 2), this line was assigned to the Fe–O stretching mode of saNOS/NOHA. The 33 cm$^{-1}$ isotopic shift was larger than that predicted for an Fe–O diatomic oscillator, but similar to the ~30 cm$^{-1}$ shifts of substrate-free saNOS and cytochrome P450$_{\text{cam}}$ (Table 2). The $v_{\text{Fe-O}}$ frequency of saNOS/NOHA was thus 13 cm$^{-1}$ higher than that of substrate-free saNOS and of saNOS complexed with l-arginine (23). (Table 2). Like the $v_{\text{Fe-O}}$ mode, the frequency of the $v_{\text{Fe-O}}$ mode was not affected by H$_4$B binding, and like saNOS and saNOS/Arg (23), the $v_{\text{Fe-O}}$ mode of Fe$^{III}$O$_2$ saNOS/NOHA was not detected. Finally, the $^{16}$O$_2$-minus-$^{18}$O$_2$ difference spectrum recorded at 81 ms after mixing was nearly flat, with a small difference near 530 cm$^{-1}$ from the $v_{\text{Fe-O}}$ mode due to the remaining 8% of the Fe$^{III}$O$_2$ complex present at 81 ms (Fig. 4, trace I).

Analysis of the resonance Raman spectra revealed that substrate and H$_4$B binding caused changes in the intensity of some lines. The intensity of the $v_{\text{Fe-O}}$ out-of-plane mode at 677 cm$^{-1}$ of the spectrum of substrate-free saNOS was much higher than that of the $v_{15}$ out-of-plane mode at 693 cm$^{-1}$ (23). As observed previously with l-arginine (23), upon binding of NOHA, the intensity of $v_{\text{Fe-O}}$ decreased and that of $v_{15}$ increased. These lines were therefore of nearly equal intensity in the presence of substrate (Fig. 4, traces A–D). Although H$_4$B did not affect the frequencies of the Fe–O and O–O stretching modes, H$_4$B binding decreased the intensity of the heme mode at 397 cm$^{-1}$ (Fig. 4, traces A–D), which is in the region where the bending modes of the heme propionates are usually observed (31). A mode at 548 cm$^{-1}$ was observed in the Fe$^{III}$O$_2$ spectra obtained with NOHA and H$_4$B (Fig. 4, traces A and B) that was absent in the spectra obtained with NOHA alone (traces C and D). The line at 548 cm$^{-1}$ likely corresponded to the $v_{\text{Fe-O}}$ mode of the small population of the Fe$^{III}$NO complex present 4.5 ms after mixing (23).

Identification of the $v_{\text{Fe-O}}$ and $v_{\text{O-O}}$ Mode Frequencies of bsNOS—We obtained the resonance Raman spectra of the oxygenated complexes of a second bacterial NOS (bsNOS) saturated with l-arginine or NOHA. Stopped-flow spectroscopy was first used to obtain the rates of formation and decay of the Fe$^{III}$O$_2$ complex of bsNOS in the presence of l-arginine (without H$_4$B) (supplemental Fig. 3) and NOHA (without H$_4$B) (sup-
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FIGURE 5. Identification of the $v_{\text{Fe-O}}$ and $v_{\text{O-O}}$ modes of bsNOS saturated with l-arginine and of bsNOS saturated with NOHA. The resonance Raman spectra were recorded 22 ms after mixing reduced bsNOS/l-arginine with $^{16}\text{O}_2$-saturated (trace A) and $^{18}\text{O}_2$-saturated (trace B) buffers ($\text{O}_2$ concentration before mixing of 40%) and 32 ms after mixing reduced bsNOS/NOHA with $^{16}\text{O}_2$-saturated (trace C) and $^{18}\text{O}_2$-saturated (trace D) buffers. The $^{18}\text{O}_2$-minus-$^{16}\text{O}_2$ difference spectra of the samples containing l-arginine (trace E) and NOHA (trace F) are shown. The excitation wavelength was 441.6 nm, and the power was 12 milliwatts.

The resonance Raman spectra of the FeIICO complexes of bsNOS/L-Arg (28) and FeIINO complexes (five-coordinate) are shown. The excitation wavelength was 441.6 nm, and the power was 12 milliwatts.

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Substrate-specific Interactions with O$_2$ in NOS

Interactions of the Heme-bound O$_2$ with l-Arginine and NOHA—We reported previously that the presence of l-arginine causes a 12 cm$^{-1}$ downshift in the v$_{O-O}$ frequency of the Fe$^{III}$O$_2$ complex of saNOS, but does not modify the frequency of the v$_{Fe-O}$ mode (23). This effect of l-arginine correlates with the increased stability of the Fe$^{III}$O$_2$ complex, which decays at rates of 5.6 and 1 s$^{-1}$ compared with 39.6 s$^{-1}$ without l-arginine (23). These results suggest that hydrogen-bonding interactions with the heme-bound O$_2$ stabilize the complex against autoxidation to the ferric form.

In the Fe$^{III}$O$_2$ complex of saNOS/NOHA presented here, the frequency of the v$_{O-O}$ mode was 1135 cm$^{-1}$, which is the same as that for substrate-free saNOS (23). The lack of sensitivity to NOHA binding of the v$_{O-O}$ frequency has already been observed with the nNOS oxygenase domain (22, 24) and very recently with the mammalian inducible nitric-oxide synthase (iNOS) oxygenase domain (35). However, unlike l-arginine, NOHA caused an upshift of the v$_{Fe-O}$ frequency from 517 to 530 cm$^{-1}$ in saNOS and bsNOS, assuming that the substrate-free protein has a v$_{Fe-O}$ mode at 517 cm$^{-1}$ like substrate-free saNOS. These results indicate that NOHA binding modifies the frequency of the v$_{Fe-O}$ mode, but not that of the v$_{O-O}$ mode, whereas l-arginine binding modifies the frequency of the v$_{O-O}$ mode, but not that of the v$_{Fe-O}$ mode.

With regard to H$_2$B, our results indicate that a pterin is required for the synthesis of NO from NOHA in saNOS, as an Fe$^{III}$NO complex was observed in single turnover experiments performed in the presence of H$_2$B (23), but not in its absence (Fig. 1). This means that H$_2$B is able to support NO synthesis with saNOS as with other bacterial NOs (28, 36), but it does not mean that H$_4$B is the natural cofactor of saNOS. The Fe$^{III}$O$_2$ intermediate formed with NOHA-saturated saNOS was the same irrespective of whether H$_2$B was present or not, as indicated by the similarity of the optical spectra (Table 1) and the same v$_{O-O}$ and v$_{Fe-O}$ frequencies (Table 2). These results suggest that H$_2$B is involved in reactions that occur after the formation of the oxygenated complex, as is the case with l-arginine (23), and are consistent with the results of Wei et al. (10, 14), who showed that an H$_2$B radical is formed only after the
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appearance of the Fe$^{3+}$O$_2$ complex in single turnover experiments with iNOS/NOHA/H$_2$B.

The divergent sensitivities of the Fe–O and O–O stretching modes to substrate (l-arginine and NOHA) binding most likely reflect different interactions of the substrates with the heme-bound O$_2$. Three types of factors can modulate the $\nu_{\text{Fe-O}}$ and $\nu_{\text{O-O}}$ frequencies: the polarity of the environment near the O$_2$, including hydrogen-bonding interactions; steric interactions; and the strength of the interaction with the sixth ligand (cysteine in NOS). Steric interactions that would change the geometry (bending and tilting) of the Fe–O–O unit could contribute to modulating the $\nu_{\text{Fe-O}}$ frequency. l-Arginine binding did not change the $\nu_{\text{Fe-O}}$ frequency or the amplitude of the isotope shift, indicating that it did not change the Fe–O–O angle (37). Oxygenated complexes are naturally bent (33), and experimental evidence with heme oxygenase indicates that constraining the Fe–O–O unit in a highly bent configuration lowers the $\nu_{\text{Fe-O}}$ frequency (37). This behavior is opposite to the 13 cm$^{-1}$ upshift of the $\nu_{\text{Fe-O}}$ frequency observed with saNOS and bsNOS upon NOHA binding. It is thus unlikely that NOHA causes a bending of the Fe–O–O unit.

The polarity effect, including hydrogen-bonding interactions of substrates with heme-bound ligand, is very likely given that hydrogen bond formation was demonstrated for nNOS and iNOS from the deuterium effect on heme-bound CO (38–40) and suggested from the crystal structures of heme-CO and heme-NO complexes of nNOS, mammalian endothelial NO (eNOS), and bsNOS (41, 42). Particularly interesting with respect to the results presented in this study are the crystal structures the Fe$^{3+}$NO complexes of bsNOS/l-arginine and bsNOS/NOHA revealing that the heme-bound NO is likely involved in hydrogen-bonding interactions with the guanidinium group of l-arginine and water for the l-arginine-bound enzyme. With NOHA, only hydrogen bonds to the $^{14}$NH of NOHA would be formed as the water molecule is displaced away from the heme (Fig. 7) (42).

Several studies from the last 10 years indicate that the $\nu_{\text{Fe-O}}$ mode is sensitive to hydrogen-bonding interactions involving the proximal oxygen atom. Indeed, the truncated hemoglobin and HemAT sensor protein display smaller frequencies of the $\nu_{\text{Fe-O}}$ mode (554–564 cm$^{-1}$) than myoglobin (570 cm$^{-1}$) in response to hydrogen bonding with distal residues (43, 44). It has been established that the hydrogen bond with the proximal oxygen (the oxygen directly ligated to the heme) causes the downshift of the $\nu_{\text{Fe-O}}$ frequency, as hydrogen-bonding interactions with the terminal oxygen, as in myoglobin, do not shift the $\nu_{\text{Fe-O}}$ frequency (43–45). The $\nu_{\text{O-O}}$ mode is also sensitive to hydrogen-bonding interactions, but unlike the $\nu_{\text{Fe-O}}$ mode, it is sensitive to hydrogen bonding involving the terminal oxygen (46).

By analogy to the truncated hemoglobins and the HemAT sensor, the upshift of the $\nu_{\text{Fe-O}}$ frequency from 517 to 530 cm$^{-1}$ upon NOHA binding could be attributed to the disruption of a hydrogen bond to the proximal oxygen, already present in substrate-free saNOS and l-arginine-bound saNOS (Fig. 7, Scheme A). This hydrogen bond would rather likely involve a water molecule(s), which is consistently observed in the heme pocket of crystal structures of NOs (41, 42) because no heme pocket group appears to be close enough to the heme-bound ligands to play that role. In Scheme A, additional hydrogen bonding to the terminal oxygen upon l-arginine binding would cause a downshift of $\nu_{\text{O-O}}$ due to modulation of n-backbonding. Because the frequency of the $\nu_{\text{O-O}}$ mode does not change upon NOHA binding with respect to substrate-free saNOS, Scheme A implies that no additional hydrogen-bonding interactions are made with the terminal oxygen. The kinetics of formation and decay of the oxygenated complex support this model, as the decay rate remains high with NOHA-bound saNOS (Table 1).

This model may also explain the low frequency of the $\nu_{\text{Fe-O}}$ mode in saNOS and bsNOS with respect to cytochrome P450$_{cam}$, which was unexpected because the Fe–Cys bond is actually weaker in NOs (Fe–Cys stretching mode at 337 cm$^{-1}$).

FIGURE 7. Interactions between heme-bound ligands and the substrates l-arginine and NOHA. Shown are the structures of the Fe$^{3+}$NO complexes of bsNOS with l-arginine (Protein Data Bank code 2FCI) (A) and NOHA (Protein Data Bank code 2FE2) (B) (42). Also shown are models of the oxygenated complexes for saNOS and bsNOS (substrate-free (C) and with l-arginine (D) and NOHA (E)) based on our resonance Raman results (water molecules are included based on A and B). In Scheme A, hydrogen bonds are formed in substrate-free NO from water to both oxygen atoms of the heme-bound O$_2$. With l-arginine, additional hydrogen-bonding interactions with the terminal oxygen increase n-back bonding from the heme iron and thus lower the $\nu_{\text{O-O}}$ frequency (~70% of the enzymes display the 1123 cm$^{-1}$ mode) (23). With NOHA, the hydrogen bond to the proximal oxygen is disrupted, thus increasing the frequency of $\nu_{\text{Fe-O}}$. In Scheme B, there is no hydrogen bond involving the heme-bound O$_2$. With l-arginine, 70% of the enzymes form strong hydrogen bonds with the heme-bound terminal O$_2$, and ~30% remain hydrogen bond-free (not shown) (23). With NOHA, all saNOS molecules form hydrogen bonds with the heme-bound O$_2$ (strong with the proximal oxygen). The hydrogen bond is drawn from $^{14}$NH of NOHA as the crystal structures in A and of mammalian NOs (61, 62) indicate that the hydroxyl is pointing away from the heme iron. The frequencies of the $\nu_{\text{Fe-O}}$ and $\nu_{\text{O-O}}$ modes (cm$^{-1}$) are indicated for oxygenated complexes (shown as Fe$^{3+}$O$_2$) in C–E.
for iNOS oxygenase domain (47), 338 cm⁻¹ for eNOS (48), and 342 cm⁻¹ with bsNOS (47)) than in P450cam (Fe–Cys at 351 cm⁻¹) (49, 50). Scheme A is, however, difficult to reconcile with the hydrogen-bonding pattern inferred for both atoms of NO from the crystal structures of the FeIII(NO) complex of bsNOS/NOHA described above, analysis of the oxygenated complex of a NOS model by density functional theory methods indicating that hydrogen-bonding interactions involving both oxygen atoms of the heme-bound O₂ are likely (13), and the discussion of Poulos and co-workers (41) describing the orbitals of NOHA that are better matched for hydrogen-bonding interactions with the heme-bound O₂ than those of L-arginine. Also from Scheme A, one has to assume that a water molecule can be stabilized in the heme pocket in the absence of substrate and make strong hydrogen bonds to the proximal and distal oxygen atoms of the heme-bound O₂.

A second scenario may be envisioned (Fig. 7, Scheme B). As discussed earlier, the proximal cysteine of thiolate-coordinated heme proteins is able to compete for the Fe₆z₂ orbitals and thus weaken the σ-bond between O₂ and iron (reduced νFe–O frequency). In both mammalian and bacterial NOSs, the proximal cysteine shares hydrogen bonds with the nitrogen atom of the side chain of a Trp and amide protons (1, 51, 52), so the electron-donating properties of the proximal cysteine may be modulated by strengthening or weakening these interactions (53, 54). Interestingly, it was recently shown that the strength of the Fe–Cys bond is modulated in response to different substrate binding in P450 (55). With these ideas in mind, we propose that the interactions between the heme-bound O₂ with NOHA or an NOHA/water network involve the formation of a strong hydrogen bond to the proximal oxygen. This hydrogen bond should decrease the νFe–O frequency. However, if the new hydrogen-bonding interactions impose a better alignment of the O₂ orbitals with the Fe₆z₂ orbitals, the proximal cysteine may become a poorer competitor for σ-bond formation with the heme iron so that the net effect could be a stronger σ-bond between the iron and O₂ (increased νFe–O frequency). This hydrogen bond network, would also stabilize Fe–O–O in a more rigid configuration that is indeed suggested by the increased sharpness of the νO–O line with NOHA present (20 cm⁻¹ in saNOS and 15.4 cm⁻¹ in bsNOS) than without NOHA (22 cm⁻¹ in saNOS). This scenario is consistent with studies by density functional theory methods indicating that hydrogen bonds to both oxygen atoms are formed in the oxygenated complex of NOS/NOHA (12). It is also consistent with the crystal structure of the FeIII(NO) complex of bsNOS discussed previously (42). With L-arginine, a strong hydrogen bond to the terminal oxygen would cause a downshift of νO–O to 1123 cm⁻¹ due to the modulation of π-backbonding (56, 57). It must be pointed out that similar but not identical EPR signals have been obtained upon cryoreduction of the FeIII(NO) complex of the eNOS oxygenase domain saturated with L-arginine and NOHA, suggesting that substrate-specific interactions within the peroxy intermediate do occur (21). An alternative scenario, i.e. that the peroxy intermediate itself hydroxylates NOHA, may also be envisaged (21). In this case, the preferred interaction of the proximal oxygen with NOHA proposed in Scheme B may be essential to stabilize the peroxy intermediate and to prevent protonation of the heme-bound O₂. Finally, in the context of Scheme A, it is the displacement of the water molecule away from the heme that could be critical for the proper oxidation of NOHA. Instead of protecting the heme-bound O₂ from protonation by hydrogen-bonding interactions as in Scheme B, the displacement of the water molecule might protect the O–O bond from being cleaved by withdrawing a source of proton(s) and by being not in direct hydrogen-bonding interactions with the proximal and distal oxygen atoms of O₂. Clearly, more studies are needed to fully understand the interactions between O₂ substrates, and water at the active site of NOSs and to obtain evidence as to whether Scheme A or B better describe those interactions.

**Implications for Catalysis**—As presented in the Introduction, the catalytic cycles of NOSs start with the reduction of the heme and the binding of O₂. In mammalian NOSs, this electron is transferred from NADPH via the reductase domain, whereas in bacterial NOSs, because they lack the reductase domain, this electron would be transferred from a separate protein (6). It is proposed that the FeIII(O₂) complex is then activated with an electron to form a peroxy complex. In this context, Scheme B, which proposes that hydrogen-bonding interactions between the heme-bound O₂ and L-arginine involve primarily the distal oxygen, would be consistent with EPR/electron nuclear double resonance investigations of the cryoreduced peroxy complexes of eNOS/L-arginine, which indicated that hydrogen-bonding interactions involving the distal oxygen atom of the peroxy complex and a water/guanidinium network occur (21). Interactions of the water/guanidinium network with the terminal oxygen of the heme peroxy are expected to favor double protonation of the distal oxygen and to ultimately lead to O–O bond scission and Compound I formation. In contrast, quantum mechanical/molecular mechanical study of the electron transfer from H₂B to the oxygenated complex in an L-arginine-bound NOS model suggests that protonation of both oxygen atoms of the oxygenated complex occurs before electron transfer to produce Compound I by a novel mechanism (9). If this is indeed how Compound I is formed in NOSs, Scheme A, which involves hydrogen bonds to both oxygen atoms of the heme-bound O₂, would be attractive, as the protonation of both oxygen atoms could be favored. All our results were obtained from the characterization of oxygenated complexes (FeIII(O₂)₂), thus offering no clue as to whether electron or protons are first transferred to activate the oxygen.

With regard to the second catalytic cycle, the formation of a hydrogen bond to the proximal oxygen of the FeIII(O₂) complex with NOHA or an NOHA/water network proposed in Scheme B could remain in the peroxy complex, thus favoring protonation of the proximal oxygen atom, which would inhibit cleavage of the O–O bond (13, 56, 57). It must be pointed out that similar but not identical EPR signals have been obtained upon cryoreduction of the FeIII(O₂) complex of the eNOS oxygenase domain saturated with L-arginine and NOHA, suggesting that substrate-specific interactions within the peroxy intermediate do occur (21). An alternative scenario, i.e. that the peroxy intermediate itself hydroxylates NOHA, may also be envisaged (21). In this case, the preferred interaction of the proximal oxygen with NOHA proposed in Scheme B may be essential to stabilize the peroxy intermediate and to prevent protonation of the heme-bound O₂. Finally, in the context of Scheme A, it is the displacement of the water molecule away from the heme that could be critical for the proper oxidation of NOHA. Instead of protecting the heme-bound O₂ from protonation by hydrogen-bonding interactions as in Scheme B, the displacement of the water molecule might protect the O–O bond from being cleaved by withdrawing a source of proton(s) and by being not in direct hydrogen-bonding interactions with the proximal and distal oxygen atoms of O₂. Clearly, more studies are needed to fully understand the interactions between O₂ substrates, and water at the active site of NOSs and to obtain evidence as to whether Scheme A or B better describe those interactions.

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