Little is known about the intermediates formed during catalysis by nitric-oxide synthase (NOS). We report here the characterization by resonance Raman spectroscopy of the oxygenated complex of the NOS from *Staphylococcus aureus* (saNOS) as well as the kinetics of formation and decay of the complex. An oxygenated complex transiently formed after mixing reduced saNOS with oxygen and decayed to the ferric enzyme with kinetics that were complex transiently formed after mixing reduced saNOS with oxygen. We report here the characterization by resonance Raman spectroscopy of the oxygenated complex. An oxygenated complex displayed a Soret absorption band centered at 430 nm. Resonance Raman spectroscopy revealed that it can be described as a ferric superoxide form (Fe$^{2+}$O$_2$$^-$) with a single $v_{o_2}$ mode at 1135 cm$^{-1}$. In the presence of L-arginine, an additional $v_{o_2}$ mode at 1123 cm$^{-1}$ was observed, indicating an increased π back-bonding electron donation to the bound oxygen induced by the substrate. With saNOS, this is the first time that the $v_{o_2}$ mode of a NOS has been observed. The low frequency of this mode, at 517 cm$^{-1}$, points to an oxygenated complex that differs from that of P450$_{cam}$. The electronic structure of the oxygenated complex and the effect of L-arginine are discussed in relation to the kinetic properties of saNOS and other NOS.

Nitric-oxide synthases (NOS) produce nitric oxide (NO) from L-arginine. In mammals, NO is essential to biological processes such as vasodilation, neurotransmission, and immune responses (1). Mammalian NOSs are homodimeric proteins (2–4). Each monomer comprises a carboxyl-terminal reductase domain where the FMN and FAD cofactors bind and an amino-terminal oxygenase domain where iron-protoporphyrin IX (heme) and tetrahydrobiopterin (H$_4$B), two prosthetic groups essential to the hydroxylation reactions, bind (2–4).

The two-step reaction involves a first hydroxylation step that converts L-arginine to the N-hydroxy-L-arginine (NOHA) intermediate and a second hydroxylation step that converts NOHA to citrulline and NO (3, 5). The active site of NOS consists of a heme coordinated to the sulfur atom of a cysteine amino acid on its proximal side and a substrate-binding pocket on the distal side (2). The H$_4$B-binding site is in the vicinity of the heme propionates and perpendicular to the heme. In mammalian NOS, H$_4$B is required to transfer an electron, and possibly a proton (6), to activate the oxygenated complex and permit L-arginine hydroxylation (4, 5, 7). Crystal structures of mammalian NOS have shown that the substrate and cofactor H$_4$B have extensive Van der Waals- and hydrogen-binding interactions with many residues as well as the heme propionates (2, 4).

NOS are present in several bacteria. Four bacterial NOSs from *Nocardia* sp. (8), *Deinococcus radiodurans* (drNOS) (9), *Bacillus subtilis* (bsNOS) (10) and *Staphylococcus aureus* (saNOS) (11, 12) have been characterized so far. Crystal structures are now available for the latter two (12, 13). Their overall structures are very similar to those of the oxygenase domain of mammalian NOS, including the substrate-binding site. However, with a shorter amino terminal hook, bacterial NOS lacks the amino-terminal hook, zinc binding site, a portion of the cofactor binding site, and some of the residues that constitute the dimer interface of mammalian NOS. drNOS, bsNOS, and saNOS produce nitrite in reconstituted enzymatic systems (9, 12). In addition, bsNOS has been shown to synthesize NO under single turnover conditions (10), indicating that it is indeed a NOS.

Recent studies have shown that saNOS, unlike mammalian NOS, does not need perin to stabilize the iron-thiolate bond (11). However, peritins such as H$_4$B and tetrahydrofolic acid interact with saNOS as shown by the conservation of the heme vibrational deformation modes in the ferric NO complexes of saNOS, nNOS, and iNOS (14, 15). Recent results have revealed that drNOS can catalyze the regiospecific nitration of tryptophan (16). Surprisingly, this activity is completely inhibited by H$_4$B, suggesting that it occurs at the cofactor site. H$_4$B may not be synthesized by bacteria that contain NOS genes (2). Nevertheless, H$_4$B and tetrahydrofolic acid can support the catalytic activities of bsNOS and drNOS (9, 10). However, the identity of the native cofactor of bacterial NOS remains to be determined.

During catalysis, oxygenated complexes of NOS must be formed (5). The oxygenated complexes (Fe$^{2+}$O$_2$$^-$) of nNOS (17–20), iNOS (21), and eNOS (22, 23) have been characterized by optical absorption spectroscopy. At least two types of oxygenated complexes seem to exist: heme-oxyl and heme-oxyl (22). Heme-oxyl has a Soret absorption band near 430 nm and an intact O–O bond, as shown by resonance Raman spectroscopy (24). Heme-oxyl has a Soret absorption band near 420 nm (22) and was identified as an oxygenated complex based on the similarity of its magnetic circular dichroism spectrum to that of P450$_{cam}$ (25). In eNOS, heme-oxyl is formed with H$_4$B present but not the substrate. Heme-oxyl is formed with the substrate present or when both the substrate and H$_4$B are absent (22). bsNOS and drNOS both form oxy-
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generated complexes with a Soret absorption band at 427 nm similar to the heme-oxyl complex of mammalian NOS (9, 10). Although it is known that heme-oxyl is an oxygenated complex, its electronic configuration is not completely understood. In particular, the Fe–O bond has never been previously observed in NOS. We report here the characterization of the oxygenated complex of saNOS by resonance Raman spectroscopy, which provides important information about the electronic properties of this complex formed during catalysis by NOS.

EXPERIMENTAL PROCEDURES

Materials—H2B, NOHA, and D2O (99.9% purity) were purchased from Sigma-Aldrich. L-Arginine was from Alexis Biochemical (San Diego, CA). Argon and 18O2 gas were from Praxair (Mississauga, Ontario, Canada). 18O2 gas (99% purity) was from Icon (Mt. Marion, NY).

Enzyme Preparation—saNOS was expressed in Escherichia coli from the cloned gene and was purified as described previously (11). Samples were maintained in 40 mM HEPES (pH 7.6), 150 mM NaCl and 1 mM dithiothreitol. Where indicated, l-arginine (1 mM or 100 μM), NOHA (500 μM), and citrulline (500 μM) were added to the purified enzyme. For samples containing H2B, a concentration of 500 μM was used. The association of cofactor H2B and substrates with saNOS were monitored by the displacement of dithiothreitol bound to the ferric enzyme using optical spectroscopy (11).

Stopped-flow Spectroscopy—Stopped-flow experiments were carried out with an Applied Photophysics (Leatherhead, UK) SX.18MV stopped-flow spectrometer installed inside an MBraun (Stratham, NH) LabMaster 100 glovebox where the oxygen concentration was maintained below 4 ppm. The dead time of the stopped-flow apparatus was 1.5 ms with the 10-mm path length cell. Anaerobic protein samples (5 ml/min) were added to the purified enzyme. The concentration of the sodium dithionite stock solution was determined by the reduction of a small aliquot of the anaerobic saNOS sample. Complete reduction was assessed by optical spectroscopy. Air-saturated buffer was used for mixing experiments with 16O2. Buffer with 20% 18O2 was prepared from buffer equilibrated with one atmosphere of 18O2 (2 ml) and diluted to 10 ml with anaerobic buffer. For the experiments with NOHA, buffers saturated with 100% 18O2 and 16O2 were used.

The rapid mixer was made anaerobic with a solution of 10 mM sodium dithionite followed by washing with anaerobic buffer to remove the excess dithionite. Two 10-ml syringes filled with oxygenated buffer and the 80 μM reduced saNOS solution, respectively, were used. The output at 441.6 nm from a He/Cd laser (Liconix laser, Melles-Griot, Ottawa, Ontario, Canada), at ~12 milliwatts was focused on the sample inside the channel of the quartz cell. The resonance Raman spectra were acquired using previously described equipment (11) at 4 ms to 100 ms after mixing, depending on the sample. Several 30-s spectra (12 to 13) were acquired and averaged. All resonance Raman spectra were obtained at room temperature (25 °C). The resonance Raman spectra were calibrated with the lines of indene. The resonance Raman spectrum of reduced myoglobin was recorded before each experiment to check for small differences between calibrations on different days. Equilibrium resonance Raman spectra were obtained as previously described (11).

RESULTS

Kinetics of Formation and Decay of the Oxygenated Complex—The kinetics of formation and decay of the oxygenated complex of saNOS were determined by stopped-flow optical spectroscopy. The results obtained by mixing oxygenated buffer with reduced saNOS, saturated with l-arginine are shown in Fig. 1. The kinetic traces at 415 nm and 440 nm are shown in Fig. S1. The efficiency of mixing was determined by the diffusion limited acid-base reaction between Na2HPO4 and HCl followed by Raman spectroscopy (27). With this method, at the first observable point after mixing, 93% of HPO4 2− was converted into H2PO4− with a flow rate of 3 ml/min−1 (supplemental data, Fig. S1). The efficiency of mixing decreased to 92% and 88% at 2 and 1 ml/min, respectively. A 3 ml/min flow rate was used thereafter. The theoretical dead time of the mixer was 0.28 ms. The actual dead time determined from the fluorescence signal generated by the formation of a hydroxyquinoline Mg2+ complex (28), was 0.49 ± 0.04 ms (supplemental data, Fig. S2).

Resonance Raman Spectroscopy with the Continuous-flow Mixer—Prior to mixing, the reduced form of saNOS (80 μM) was prepared by equilibrating the enzyme with pure argon gas for at least 30 min at room temperature and by adding 1.25 molar equivalents of sodium dithionite. The concentration of the sodium dithionite stock solution was determined by the reduction of a small aliquot of the anaerobic saNOS sample. Complete reduction was assessed by optical spectroscopy. Air-saturated buffer was used for mixing experiments with 16O2. Buffer with 20% 18O2 was prepared from buffer equilibrated with one atmosphere of 18O2 (2 ml) and diluted to 10 ml with anaerobic buffer. For the experiments with NOHA, buffers saturated with 100% 18O2 and 16O2 were used.

The rapid mixer was made anaerobic with a solution of 10 mM sodium dithionite followed by washing with anaerobic buffer to remove the excess dithionite. Two 10-ml syringes filled with oxygenated buffer and the 80 μM reduced saNOS solution, respectively, were used. The output at 441.6 nm from a He/Cd laser (Liconix laser, Melles-Griot, Ottawa, Ontario, Canada), at ~12 milliwatts was focused on the sample inside the channel of the quartz cell. The resonance Raman spectra were acquired using previously described equipment (11) at 4 ms to 100 ms after mixing, depending on the sample. Several 30-s spectra (12 to 13) were acquired and averaged. All resonance Raman spectra were obtained at room temperature (25 °C). The resonance Raman spectra were calibrated with the lines of indene. The resonance Raman spectrum of reduced myoglobin was recorded before each experiment to check for small differences between calibrations on different days. Equilibrium resonance Raman spectra were obtained as previously described (11).
FIGURE 1. Kinetics of formation and decay of the oxygenated complex of saNOS in the presence of L-arginine. A, variation in the absorbance at 415 and 440 nm (dotted lines) and the fits to the three-species kinetic model (solid lines); B, calculated spectra of the reduced (solid line), the oxygenated (dotted/dashed line), and the ferric forms (dotted line); C, variations in the concentrations of the three species as a function of time. D, residuals of the fits at all wavelengths as a function of time. All the residuals showed \( \Delta A < 0.0015 \), indicating a good fit of the data to the three-species kinetic model. The L-arginine concentration was 100 \( \mu M \).

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TABLE 1

Rates of formation and decay of the oxygenated complexes of saNOS, drNOS, and bsNOS and the wavelength maxima of the Soret absorption band of the oxygenated complexes

| Protein                  | Formation of \( Fe^{II}\)O\(_2\) | Decay rate* (fraction) | Soret   | Temp. | Ref. |
|--------------------------|-------------------------------|------------------------|--------|-------|------|
| saNOS\(^b\)              | 1880                          | 39.6 (100%)            | 430    | 21    | This work |
| saNOS/Arg\(^b\)          | 106                           | 5.6 (14%) 1.0 (86%)    | 430    | 21    | This work |
| saNOS/H\(_4\)B\(^b\)     | 2080                          | 32.8 (78%) 3.6 (22%)   | 425, 430\(^d\) | 21    | This work |
| saNOS/Arg/H\(_4\)B\(^b\) | 143                           | 18.0 (67%) 5.0 (33%)   | 405, 430 | 21    | This work |
| saNOS/NOHA/H\(_4\)B\(^c\) | 454                           | 22.2 (100%)            | 428    | 21    | This work |
| drNOS/Arg\(^d\)          | 72                            | 1.37                   | 427    | 10    | 9    |
| drNOS/H\(_4\)B/Arg\(^d\) | 60                            | 3.9                    | 427    | 10    | 9    |
| bsNOS/Arg\(^d\)          | 60                            | 0.4                    | 427    | 10    | 10   |
| bsNOS/Arg/H\(_4\)B\(^d\) | 80                            | 5.4                    | 427    | 10    | 10   |

*The rate of decay corresponds to autoxidation when catalysis cannot occur: \( -\text{Arg}^+/H\(_4\)B + \text{Arg}^+/H\(_4\)B \) and \( -\text{Arg}^+/H\(_4\)B \). The rate of decay corresponds to oxygen activation when \( \text{L-arginine} \) or NOHA are present with H\(_4\)B.

\( \Delta \lambda \) nm and the fit to the three species kinetic model are shown in Fig. 1A. These wavelengths show the largest variations of absorbance. The good fit of the data to the kinetic model was shown by the very small amplitude of the residuals at all wavelengths (<0.0015 \( \Delta \lambda \)) over the course of the reaction (Fig. 1D).

The deconvoluted absorption spectra of the reduced, oxygenated, and oxidized species were obtained (Fig. 1B). Reduced saNOS (solid line) displayed an absorption maximum at 412 nm, just like the spectrum of the starting material before mixing (11). The transiently formed oxygenated species (dashed/dotted line) displayed a Soret absorption maximum at 430 nm, whereas the oxidized form (dotted line), which is the species obtained at the end of the kinetic reaction, displayed a Soret absorption band centered at 398 nm. Because L-arginine was bound at the active site, the ferric spectrum was that of the five-coordinate, high spin form (11).

The variation in concentration of the three species as a function of time is shown in Fig. 1C. The oxygenated complex reached a maximal concentration of 2.4 \( \mu M \) (96% of the total heme concentration) 15–30 ms after mixing. The rate of formation of the oxygenated complex was 106 s\(^{-1}\) (Table 1). Subsequently, the oxygenated species was converted to the oxidized form, with rate constants of 5.6 s\(^{-1}\) and 1 s\(^{-1}\), respectively (Table 1).

In the absence of substrate, the appearance and decay of the oxygenated complex were faster, and the complex reached a concentration of 2.3 \( \mu M \) 2.1 ms after mixing (supplemental data, Fig. S3). The rate of formation of the oxygenated complex was 1880 s\(^{-1}\) (Table 1). This complex decayed with a single rate constant of 39.6 s\(^{-1}\) (Table 1).

The addition of NOHA or H\(_4\)B did not affect the rate of formation of the oxygenated complex, which remained at 2080 s\(^{-1}\) (Table 1). With both L-arginine and H\(_4\)B, the rate of formation of the oxygenated complex (143 s\(^{-1}\)) was similar to that with L-arginine alone (106 s\(^{-1}\)). Although H\(_4\)B had only a small effect on the rate of formation of the oxygenated complex, it increased the rate of decay in the presence of L-arginine, with rates of 18 and 5 s\(^{-1}\), respectively, compared with rates of 5.6 and

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1.0 s\(^{-1}\) with L-arginine alone (Table 1). In the absence of L-arginine, H4B slowed the decay from a single rate of 39.6 s\(^{-1}\) to two rates of 32.8 and 3.6 s\(^{-1}\), respectively (Table 1).

**Optical Spectra of the Oxygenated Complexes**—For every condition, an oxygenated species with a Soret absorption band centered at 430 nm was formed (Table 1). For samples with no H4B, this was the only oxygenated species with a Soret absorption band centered at 430 nm (Table 1). The formation and decay of the two oxygenated species over the same time scale or a fraction of one species was characterized by resonance Raman spectroscopy.

For the sample containing both L-arginine and H4B, the kinetic model included three species. The deconvoluted oxygenated spectrum had a wide Soret absorption band consisting of two peaks centered approximately at 405 and 430 nm (Table 1). These species could not be resolved by including a fourth species in the kinetic model. Either both oxygenated species formed over the same time scale or a fraction of one species was converted to the other very rapidly before the slow decay to the ferric form. As the laser used for resonance Raman spectroscopy emitted light at 442 nm, only the 430 nm species was characterized by resonance Raman spectroscopy.

**Identification of the \(\nu_{O-O}\) Mode**—To identify the vibrational modes that involved the oxygen molecule, the spectra of the oxygenated complex of saNOS were obtained with \(^{18}\text{O}_2\) and \(^{16}\text{O}_2\). The resonance Raman spectrum of the oxygenated complex of L-arginine-free and pterin-free saNOS was obtained with \(^{18}\text{O}_2\) (Fig. 3A). The background signal from the quartz flow cell, shown in Fig. 3F, was acquired and subtracted from the raw spectrum shown in Fig. 3A to obtain the spectrum shown in Fig. 3B. The spectra C–E were also corrected to remove the quartz signal. An isotopic shift from 1135 cm\(^{-1}\) to 1071 cm\(^{-1}\) was observed with \(^{16}\text{O}_2\) and \(^{18}\text{O}_2\), respectively (Fig. 3, B and C). The \(^{16}\text{O}_2\) minus \(^{18}\text{O}_2\) difference spectrum shows this shift more clearly (Fig. 4A). The 64-cm\(^{-1}\) downshift was on par with the theoretical value (\(\Delta\nu_{O-O} = 65\) cm\(^{-1}\)) expected for a diatomic harmonic oscillator. The vibrational mode at 1135 cm\(^{-1}\) was thus assigned to the \(\nu_{O-O}\) stretching mode.

With L-arginine present, the main line for the \(\nu_{O-O}\) mode was at 1123 cm\(^{-1}\) (Fig. 3D). The 61-cm\(^{-1}\) downshift to 1062 cm\(^{-1}\) with \(^{18}\text{O}_2\) (Figs. 3A and 4A) supports the assignment of the \(\nu_{O-O}\) mode to the stretching vibration of the oxygen molecule.
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Identification of the $v_{\text{Fe-O}}$ Mode of saNOS—The $^{18}\text{O}_2$ minus $^{16}\text{O}_2$ differential spectra of oxygenated saNOS presented in Fig. 4 (A–E) revealed the presence of a second isotope-sensitive vibrational mode. The line at 517 cm$^{-1}$ in the $^{18}\text{O}_2$ samples shifted to 487 cm$^{-1}$ in the $^{16}\text{O}_2$ samples. The 30-cm$^{-1}$ isotopic shift and the similar frequencies displayed by the $v_{\text{Fe-O}}$ mode of P450$_{cam}$ (Table 2) and thiolate coordinated heme models (31) indicated that this line corresponded to the $v_{\text{Fe-O}}$ mode of saNOS (Table 2). The 30-cm$^{-1}$ isotopic shift displayed by saNOS was larger than the theoretical value of 23 cm$^{-1}$ expected for a simple Fe–O diatomic oscillator. However, isotopic shifts of 27 to 30 cm$^{-1}$ have been observed for the $v_{\text{Fe-O}}$ mode of P450$_{cam}$ (Table 2). Unlike the $v_{\text{O-O}}$ mode, the $v_{\text{Fe-O}}$ mode was unaffected by the addition of L-arginine and H$_4$B (Fig. 4, A–E).

The $v_{\text{O-O}}$ lines were analyzed by spectral deconvolution. In the absence of L-arginine and pterin, the $v_{\text{O-O}}$ lines were fitted to single Gaussian peaks of similar intensity and width at 1135 and 1071 cm$^{-1}$, respectively (Fig. 5A). In all other samples, the lines were best fitted to two peaks of mixed Gaussian and Laurentzian character. H$_4$B alone had a small effect on the $v_{\text{O-O}}$ frequency as seen by the appearance of small isotopic shift and the similar frequencies displayed by the $v_{\text{Fe-O}}$ mode of P450$_{cam}$ (Table 2) and thiolate coordinated heme models (31) indicated that this line corresponded to the $v_{\text{Fe-O}}$ mode of saNOS (Table 2). The 30-cm$^{-1}$ isotopic shift displayed by saNOS was larger than the theoretical value of 23 cm$^{-1}$ expected for a simple Fe–O diatomic oscillator. However, isotopic shifts of 27 to 30 cm$^{-1}$ have been observed for the $v_{\text{Fe-O}}$ mode of P450$_{cam}$ (Table 2). Unlike the $v_{\text{O-O}}$ mode, the $v_{\text{Fe-O}}$ mode was unaffected by the addition of L-arginine and H$_4$B (Fig. 4, A–E).

To determine whether the heme-bond dioxygen was involved in hydrogen-bond interactions, the resonance Raman spectra of the oxygenated complexes of substrate-free and pterin-free saNOS were obtained in H$_2$O and D$_2$O. As shown in Fig. 4F, the H$_2$O minus D$_2$O difference spectrum displayed no shift of vibrational modes.

To detect the $v_{\text{Fe-O}}$ mode, which is observed at 402 cm$^{-1}$ for P450$_{cam}$ (32), we obtained the 200- to 900-cm$^{-1}$ region of the resonance Raman spectra of oxygenated saNOS in complex with L-arginine (supplemental data, Fig. S4). No isotope-sensitive vibrational mode other than the $v_{\text{Fe-O}}$ mode could be observed.

Synthesis of NO under Single Turnover Conditions—The hydroxylation of NOHA by saNOS containing H$_4$B was followed by stopped-flow spectroscopy under single turnover condition. Fig. 6 shows the results of the global analysis of the kinetic scanning data in the Soret and visible regions of the spectra. The reduced species, with a Soret absorption maximum centered at 413 nm (Fig. 6C, solid line) rapidly bound dioxygen at a rate of 454 s$^{-1}$ and formed the oxygenated complex with a Soret absorption band centered at 428 nm (Fig. 6C, dashed trace). The oxygenated complex was then converted to the Fe$^{III}$NO complex (Fig. 6C, dotted line) that reached a maximal concentration 100 ms after mixing (Fig. 6E). The rate at which the oxygenated complex was converted to the Fe$^{III}$NO complex was 22.2 s$^{-1}$ (Table 1).

The rapid formation of the Fe$^{III}$NO complex was clearly observed at 445 nm with a kinetic trace shifted to a longer time scale with respect to the kinetic trace at 430 nm that monitored the formation of the oxygenated complex (Fig. 6B). The Fe$^{III}$NO complex was identified by the Soret...
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### TABLE 2

| Protein              | \(v_{\text{Fe-O}}^{(18\text{O})}\) \(\text{cm}^{-1}\) | \(v_{\text{O-O}}^{(18\text{O})}\) \(\text{cm}^{-1}\) | Ref.       |
|----------------------|----------------------------------|----------------------------------|-----------|
| saNOS                | 517 (487)                        | 1135 (1071)                      | This work |
| saNOS/Arg            | 517 (487)                        | 1123 (1062) 1135 (1071)          | This work |
| saNOS/H₂B            | 517 (487)                        | 1123 (1062) 1135 (1071)          | This work |
| saNOS/Arg/H₂B        | 517 (487)                        | 1123 (1062) 1135 (1071)          | This work |
| nNOSox               | –                                | 1135 (1068)                      | 24, 40    |
| nNOSox/Arg           | –                                | 1135 (1068)~1122 (~1056)         | 24, 40    |
| nNOSox/H₂B           | –                                | 1135 (1068)                      | 24, 40    |
| nNOSox/Arg/H₂B       | –                                | 1135 (1068)~1122 (~1056)         | 24, 40    |
| P450cam/adamantanone | 541 (511)                        | 1140 (1074)                      | 29, 48    |
| P450cam/camphor      | 537 (510)                        | 1139 (1074) 1147 (1080)          | 29        |
| D251N P450cam        | 536 (505)                        | 1128 (1061) 1136 (1070)          | 38        |
| D251N P450cam/putidaredoxin | 521 (487) 536 (505) | 1129 (1062) 1137 (1071) | 38        |

a For the \(v_{\text{O-O}}\) modes of saNOS, the mode that is predominant is indicated in boldface.

b ~ absent.

c Minor fraction.

**FIGURE 6.** Synthesis of NO during a single turnover experiment with NOHA and H₂B. Reduced saNOS, saturated with 500 μM NOHA and 500 μM H₂B, was mixed with O₂-saturated buffer in the stopped-flow apparatus. A, the optical spectra were recorded between 1 ms and 1 s after mixing. Highlighted are the spectrum at 3 ms after mixing (solid line), the spectrum at 13 ms after mixing (dashed line), the spectrum at 53 ms (dotted line), and the spectrum at 860 ms (dashed/dotted line); B, the kinetic traces at 430 and 445 nm (dotted lines) and the fits to the four-species model (solid lines); C and D, calculated spectra in the Soret region (C) and in the 520- to 600-nm region (D) of the reduced (solid line), oxygenated (dashed line), FeIIINO (dotted line), and of the ferric (dotted/dashed line) forms; E, time course of the variation of the concentration of the four species.

absorption band centered at 438 nm (Fig. 6C, dotted trace) and maxima at 550 and 580 nm in the visible region (Fig. 6D, dotted trace).3

The FeIIINO complex decayed to the ferric enzyme with the Soret absorption maximum at 398 nm (Fig. 6C, dotted/dashed trace) over 1 s with a rate constant of 3.9 s⁻¹ (Fig. 6E, dotted/dashed trace) (Table 1). Fig. 6A shows the raw optical spectra highlighting the spectrum at 3 ms (solid line), a spectrum at 13 ms indicating the formation of the oxygenated complex (dashed line), a spectrum at 53 ms showing a shift to longer wavelengths indicating the formation of the FeIIINO complex (dotted line), and the final ferric spectrum (dotted/dashed line).

To confirm the formation of the FeIIINO complex, the resonance Raman spectrum was acquired 100 ms after mixing ferrous H₂B and NOHA saturated saNOS, with oxygen under single turnover condition. Stopped-flow results indicate that at 100 ms after mixing, the FeIIINO complex had reached a maximum concentration of 1.7 μM (69% of the heme total). The remaining fractions were the oxygenated (11%) and ferric forms (20%). The resonance spectrum recorded 100 ms after mixing displayed an intense vibrational mode at 547 cm⁻¹ (Fig. 7E) that we assigned to a \(\delta_{\text{Fe-N-O}}\) mode based on the comparison of this mode with that displayed by equilibrium FeIIINO forms of saNOS with and without substrates and cofactor.4 It must be pointed out that the NO synthesized by saNOS cannot be labeled using 18O₂, because the oxygen atom of NO comes from NOHA itself.

We emphasize that ferric (Fig. 7G) and ferrous forms (Fig. 7H) of saNOS do not display a resonance Raman line at 547 cm⁻¹. Also consistent with the assignment of the 547 cm⁻¹ line as a originating from the FeIIINO complex is the spectrum recorded at 5 ms after mixing (Fig. 7F), that displays a less intense line at 547 cm⁻¹ as compared with the spectrum obtained at 100 ms after mixing (Fig. 7E). Based on the stopped-flow analysis (Fig. 6E), at 5 ms after mixing, 7% of the heme content was in the FeIIINO form, 83% in the oxygenated form, and 10% in the reduced form. Accordingly, the spectrum at 5 ms displays 18O₂-sensitive vibrational modes (result not shown) and lines at 510 and 692 cm⁻¹ that originate from the oxygenated and reduced species (Fig.
Effect of l-arginine and H4B on the Rate of Decay of the Oxygenated Complex—The comparison of the autoxidation rates of l-arginine-free and pterin-free saNOS with the sample containing l-arginine shows that l-arginine slowed the autoxidation rate of the Fe\textsuperscript{II}O\textsubscript{2} complex 12- to 40-fold. A stabilization of the oxygenated complex by l-arginine has also been observed with nNOS\textsubscript{ox} (17, 18) and eNOS\textsubscript{ox} (22, 33). For eNOS\textsubscript{ox}, the slower rate of autoxidation with l-arginine has been suggested as favoring coupling with the electron transfer from H4B to activate the oxygen before the release of superoxide occurs (33).

The fast rate of formation and autoxidation of the Fe\textsuperscript{II}O\textsubscript{2} complex displayed by saNOS may result in a significant release of superoxide, with potent deleterious effects. In fact, Berk et al. (33) reported that superoxide is generated at a level of 0.3 spin/heme with saNOS in the absence of l-arginine and H4B. This is comparable with the 0.5 spin/heme produced by eNOS\textsubscript{ox} under the same conditions (33). l-Arginine helps by slowing the autoxidation rate.

H4B can transfer an electron to activate the heme-bound oxygen molecule in mammalian NOS. In saNOS, the observation of a 3- to 5-fold faster rate of disappearance for the Fe\textsuperscript{II}O\textsubscript{2} complex in the presence of both H4B and l-arginine with respect to the autoxidation rate observed with l-arginine alone is consistent with a redox function of H4B even though another pterin may be the native cofactor of the enzyme. The redox function of H4B observed with saNOS was similar to that observed with nNOS\textsubscript{ox} (17) and eNOS\textsubscript{ox} (22) with 70- and 4-fold increases, respectively, of the rate of disappearance of the Fe\textsuperscript{II}O\textsubscript{2} complex induced by H4B. Wei et al. (34) established that the electron transfer from H4B to the Fe\textsuperscript{II}O\textsubscript{2} intermediate is the rate-limiting step for all subsequent events in the single turnover hydroxylation reaction of l-arginine for all three mammalian NOS. For saNOS, like mammalian NOS, a decrease in the autoxidation rate following l-arginine binding would help couple electron transfer to oxygen activation if electron transfer from H4B and from the native cofactor is relatively slow.

**NO Synthesis**—The three mammalian NOS (6, 35, 36), as well as the bacterial NOS from *Bacillus subtilis* (10), synthesize NO in single turnover reactions. The hydroxylation of NOHA by saNOS under single turnover conditions described here shows that saNOS was able to couple electron transfer from H4B to oxygen activation and catalyze the NO synthesis reaction. NO was detected as a complex with the ferric enzyme based on the typical optical spectrum of the Fe\textsuperscript{II}O\textsubscript{2} complex observed transiently during the course of the reaction. The Fe\textsuperscript{II}O\textsubscript{2} complex appeared after the formation of the oxygenated complex and before the formation of the ferric, ligand-free enzyme. This sequence of events is exactly the same as that observed for iNOS (36), nNOS (35), and bsNOS (10) in single turnover reactions monitored by stopped-flow spectroscopy. The synthesis of NO by saNOS was confirmed by the observation of the \(\text{Fe}^{II}O\text{O}_{2}\) intermediate at 547 cm\(^{-1}\) 100 ms after mixing ferrous saNOS/H4B/NOHA with oxygen.

**Identification of the Oxygenated Complex of saNOS**—In all conditions tested, an oxygenated spectrum with a wavelength maximum of the Soret absorption band at 430 nm was observed. This oxygenated species corresponded to heme-oxy\textsubscript{II} as defined by Marchal et al. (22). The high frequency region of the resonance Raman spectrum of the oxygenated complex of saNOS indicates that the heme iron is ferric with a \(v_{1}\) line at 1341 cm\(^{-1}\) (21). The complex is low spin as shown by the \(v_{0}\) line at 1373 cm\(^{-1}\) (24, 29). In the complex with NOHA, the \(v_{0}\) line at 1500 cm\(^{-1}\) was broadened and shifted to 1555 cm\(^{-1}\). The frequency of the \(v_{1}\) line was 1435 cm\(^{-1}\) for the oxygenated complex of saNOS with a Soret absorption maximum centered at 430 nm corresponded to a heme-oxy\textsubscript{II} complex with a ferric superoxide (Fe\textsuperscript{II}O\textsubscript{2}\textsuperscript{-}) character. The oxygen-
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The oxygenated complex of saNOS was thus very similar to the heme-oxyl complex of nNOSox, which is six-coordinate, displays a FeIII-O2 character, and has a Soret absorption band at 427 nm (24).

An additional species with a blue-shifted Soret absorption maximum at 425 nm was observed with H4B alone and a species with a Soret absorption maximum at ~405 nm was observed with H4B and L-arginine combined. These species differ from the heme-oxyl species described by Marchal et al. (22), which has a Soret absorption maximum at ~420 nm. The 425 nm and the 405 nm intermediates of saNOS were not further characterized here.

The Effect of L-arginine and H4B on the O–O Stretching Mode—The binding of L-arginine caused the appearance of a new νO-O mode at 1123 cm⁻¹ in addition to the νO-O mode at 1135 cm⁻¹. Our analyses revealed that a majority of saNOS proteins adopted the new conformation in the presence of L-arginine. With P450cam, multiple νO-O frequencies of the oxygenated complex are attributed to heterogeneity conferred by the substrate (29) or to interactions with putidaredoxin (38).

With saNOS, we propose that the oxygenated complex adopts two conformations in equilibrium with each other and that L-arginine favors the conformation with the νO-O line at 1123 cm⁻¹. A conformational equilibrium is consistent with the small fraction of the form with the 1123 cm⁻¹ line detected with H4B alone. Another possibility is that the shift of the νO-O mode to 1123 cm⁻¹ reflects the hydrogen bond interaction between the oxygen molecule and L-arginine. The presence of a guanidinium group near the terminal oxygen could further polarize the Fe–O–O unit toward the ferric superoxide form. At the same time, the hydrogen bond interaction would stabilize the heme-bound superoxide and decrease the rate at which it is released from the heme. This interpretation would explain the 12- to 40-fold reduction in the rate of autoxidation of the oxygenated complex by L-arginine. In this case, the appearance of the 1123 cm⁻¹ νO-O mode with H4B alone would mean that a water molecule and/or residue of the heme pocket could mimic the effect of L-arginine in substrate-free, H4B-bound saNOS. In this respect, we have shown that with CO, a fraction of the saNOS proteins (11), like nNOSox (39), had a νFe-O mode in the absence of L-arginine at a frequency similar to that observed in the presence of L-arginine. This observation led to the proposal that a residue or water molecule of the heme pocket may be responsible for the interaction with CO.

The adoption of the conformation with the νO-O mode at 1123 cm⁻¹ seemed further enhanced by H4B in combination with L-arginine. The effect was either additive or might be caused by small changes in the conformation of the enzyme that lead to changes in the heme pocket, which in turn favor the formation of the hydrogen bond between the substrate and the heme-bound dioxygen molecule. This effect could occur through the hydrogen bond interaction that both molecules form with the heme propionate-7,9.

In the mammalian nNOSox, binding of L-arginine causes the splitting of the νO-O mode at 1135 cm⁻¹ to two vibrational modes at ~1122 and 1135 cm⁻¹, respectively (40), which is similar to our observations with saNOS. However, the fraction of nNOSox that adopts the new conformation is much lower than with saNOS, because the majority of the νO-O line remains at 1135 cm⁻¹. We conclude that the response to L-arginine binding by saNOS and nNOSox is similar, the difference being the amount of the form with νO-O at 1123 cm⁻¹ that is present when L-arginine is bound to the proteins.

Low Frequency of the Fe–O Stretching Mode—Compared with P450cam (29, 38) and thiolate model compounds (29), the frequency of the νFe-O mode of saNOS at 517 cm⁻¹ was low (Table 2). Several possibilities were considered to explain this observation.

We considered the possibility that a highly bent Fe–O–O structure would make the νFe–O–O mode appear at a low frequency. A highly bent structure, such as that displayed by heme oxygenase, would behave as an Fe–O diatomic oscillator (41). This should lead to a larger 18O2 isotopic shift of the νFe–O mode than a linear structure, which would behave like an Fe–OO oscillator (41). The isotopic shift calculated for saNOS (30 cm⁻¹) was indeed large. It was in fact larger than the expected shift for an Fe–O–O, two-body oscillator (23 cm⁻¹). However, the oxygenated complex of P450cam also displays large isotopic shifts (27–30 cm⁻¹), Table 2, and the crystal structures of the oxygenated complex show a normal Fe–O–O bending angle (129–133°) for a heme protein (42, 43).

Hydrogen bond interactions with the heme-bound proximal oxygen reduce the frequency of the νFe–O–O mode of some globins (44–46). The resonance Raman spectrum of the oxygenated complex of saNOS in D2O showed no shift of vibrational modes with respect to the spectrum in H2O. This indicates that a hydrogen bond interaction with a water molecule is unlikely. However this result does not totally rule out a hydrogen bond with an amino acid residue if the proton involved is not exchangeable. However, hydrogen bond interactions with an amino acid would require a rearrangement of the heme pocket, because there are no residues within 6 Å of the heme-Fe in saNOS (12). A hydrogen bond with the proximal oxygen would possibly favor protonation of the proximal oxygen and thus promote synthesis of peroxide and uncoupling. A hydrogen bond to the proximal oxygen thus seems unlikely.

Alternatively, we looked at electronic effects on the Fe–O–O complex. There is a well established negative correlation of the νFe–O–O modes for the Fe6NO and Fe5CO complexes of heme proteins (31, 47). This negative correlation holds true for 5-coordinate FeO2 complexes but may not hold for six-coordinate Fe6O2 complexes of thiolate-coordinated compounds. Oxygen has two electrons that can be accommodated in a π* nonbonding lone-pair orbital that bends the Fe–O–O unit or in the σ bonding orbital that retains a more linear conformation (31). Depending on the thiolate hybridization state, σ competition for the Fe dπ² orbital by the thiolate may be strong, forcing the two electrons of the O2 to the π* antibonding orbital (38). This would bend the Fe–O–O bond and lower the νFe–O frequency. If the competition is strong enough, it could result in a weak negative or even a positive correlation between the νFe–O and νO–O frequencies (38). In fact, a positive correlation between the νFe–O and νO–O frequencies has been...
observed for six-coordinate thiolate-coordinated model compounds (see Table 2 of Vogel et al. (31)), P450_camb and the P450_camb D251N-putidaredoxin complex (29, 38) (Fig. 8). The frequencies of saNOS fit with the positive correlation between the νFe–O and νO–O frequencies for 6C complexes with a thiolate bond (Fig. 8).

The low frequency of the νFe–O mode of saNOS may thus be explained by strong σ competition for the Fe d3 orbital by the thiolate, which would result in a weakening of the Fe–O bond, whereas the lower frequency of the νO–O mode of saNOS with respect to P450_camb may be explained by the extent of π back-bonding to the heme-bound dioxygen. It must be pointed out that the frequencies of the νO–O mode of nNOSox with L-arginine at 1135 cm⁻¹ and 1122 cm⁻¹ (24) are nearly identical to those of saNOS with L-arginine (Table 2). It will be interesting to see whether mammalian NOS also display a week Fe–O bond.

Although L-arginine caused a shift to a lower frequency of the νO–O mode, indicating an increase in π back-bonding, no corresponding shift of the νFe–O mode was observed. The hydrogen bond between L-arginine and the superoxide, or the indirect structural stabilizing effect of L-arginine, increases the extent of π back-bonding, thereby lowering the frequency of the O–O bond. If only π back-bonding is considered, this should make the Fe–O bond stronger. Because the νFe–O mode did not change in frequency, this indicates that σ competition by the thiolate increased with L-arginine present and was thus coordinated with the increase in the amount of π electron donation. Importantly, our results show that the reduction of the rate of autoxidation of the oxygenated complex by L-arginine did not arise from the strengthening of the Fe–O bond per se, because its frequency did not change with or without L-arginine.

Implications for Catalysis—The electronic structure displayed by saNOS with a weak Fe–O bond and a strong superoxide character may come at the price of a high rate of autoxidation of the oxygenated complex. The oxygenated complexes of saNOS and mammalian NOS are stabilized by L-arginine. However, the still quite high rate of autoxidation makes the presence of an electron donor close to the heme necessary to disfavor the autoxidation of the complex and favor oxygen activation (5, 7, 20, 39). H₂B plays this role in mammalian NOS. In saNOS, the rate of electron transfer to the heme remains to be determined, but unless it is very significantly faster than in mammalian NOS, an electron donor molecule at the cofactor site would be critical.

How the increase in the superoxide character of the heme-bound oxygen upon L-arginine binding affects the subsequent steps of oxygen activation is unclear. It is interesting that the interaction of the D251N mutant of P450_camb with putidaredoxin increases the amount of a population of the oxygenated complex with lower νO–O and νFe–O frequencies (38) (Table 2). This structural perturbation was proposed to be related to the function of putidaredoxin, which is the electron donor of P450_camb, and to involve an alteration of the electron-donating properties of the thiolate ligand.

CONCLUSIONS

We have shown that the formation and decay of the oxygenated complex of saNOS followed kinetics similar to those of mammalian NOS. L-Arginine reduced the rate of autoxidation of the oxygenated complex as with nNOSox and eNOSox. H₂B, by providing an electron for oxygen activation, increased the rate of disappearance of the oxygenated complex in the presence of L-arginine and NOHA. saNOS could synthesize NO in a single turnover reaction from NOHA. The optical spectrum of the oxygenated complex, with a Soret absorption band at 430 nm, was similar to the heme-oxysulfhydryl complex of nNOSox and eNOSox. In addition, nNOSox and saNOS display νO–O modes at nearly identical frequencies. Therefore, the properties of the oxygenated complex of saNOS that we describe here might also provide insights into the properties of the oxygenated complex of mammalian NOS.

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