Structure and reactivity of a thermostable prokaryotic Nitric Oxide Synthase that forms a long-lived oxy-heme complex.

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In an effort to generate more stable reaction intermediates involved in substrate oxidation by nitric oxide synthases (NOSs), we have cloned, expressed and characterized a thermostable NOS homolog from the thermophilic bacterium Geobacillus stearothermophilus (gsNOS). As expected, gsNOS forms nitric oxide (NO) from L-arginine via the stable intermediate Nω-hydroxy L-arginine (NOHA). Addition of oxygen to ferrous gsNOS results in long-lived heme-oxy complexes in the presence (Soret peak 427 nm) and absence (Soret peak 413 nm) of substrates L-arginine and NOHA. The substrate induced red shift correlates with hydrogen bonding between substrate and heme-bound oxygen resulting in conversion to a ferric heme-superoxy species. In single turnover experiments with NOHA, NO forms only in the presence of H4B. The crystal structure of gsNOS at 3.2 Å resolution reveals great similarity to other known bacterial NOS structures, with the exception of differences in the distal heme pocket, close to the oxygen binding site. In particular, a Lys356 (B. subtilis NOS) to Arg365 (gsNOS) substitution alters the conformation of a conserved Asp carboxylate, resulting in movement of an Ile residue closer to the heme. Thus, a more constrained heme-pocket may slow ligand dissociation and increase the lifetime of heme-bound oxygen to seconds at 4 °C. Similarly, the ferric-heme NO complex is also stabilized in gsNOS. The slow kinetics of gsNOS offer promise for studying downstream intermediates involved in substrate oxidation.

Nitric Oxide Synthases (NOSs) are highly regulated proteins that catalyze the two-step oxidation of L-arginine to nitric oxide (NO) and citrulline via the stable intermediate Nω-hydroxy L-arginine (1-3). NO functions in mammals as a potent signaling molecule and a cytotoxic agent to protect against pathogens. Mammalian NOSs consist of a reductase domain that has binding sites for FAD, FMN and NADPH and an oxygenase domain that binds iron protoporphyrin IX (heme), substrate L-arginine and the cofactor (6R)-5,6,7,8-tetrahydro-L-biopterin (H4B) (1-3). Proteins similar to mammalian NOSs have been found in a variety of lower eukaryotes including insects, fungi (4-7) and bacteria (8,9). Genes responsible for cofactor (H4B) biosynthesis are present in bacteria such as B. subtilis, G. kaustophilus, but not in other NOS-containing bacteria such as D. radiodurans (9-11). Bacterial NOS-like proteins are similar to the oxygenase domain of mammalian NOSs but contain no associated reductase module. Reductase partners for bacterial NOSs are yet to be discovered. NOSs from Deinococcus radiodurans, Bacillus subtilis, Staphylococcus aureus and Bacillus anthrasis are well characterized and have been shown to produce nitrogen oxides (NOx) in vitro (11-14). Conservation of nearly all the key residues involved in substrate and cofactor binding among mammalian and bacterial NOSs (11,13,15) suggests a similar mechanism of NO formation in the two classes of proteins.
Interestingly, *D. radiodurans* NOS can support L-arg-based NO\textsubscript{\text{a}} formation with cofactors other than H\textsubscript{\text{a}}B, such as the ubiquitous cofactor tetrahydrofolate and even tryptophan (11,16). The ability to react with L-tryptophan may be significant as NOSs from certain *Streptomyces* strains participate in biosynthetic tryptophan nitration (17).

The NOS reaction sequence is well understood (Fig. 1), although the nature of the heme-oxygen complexes directly involved in substrate oxidation remains largely unknown (18). L-arginine is first hydroxylated at the guanidino nitrogen and then the resultant N\textsuperscript{\text{\textomega}}-hydroxy-L-arginine (NOHA), an enzyme bound intermediate (19), is further oxidized to NO and citrulline. In both the arginine and NOHA reactions, reduction of the Fe(II) heme enables oxygen binding and formation of a heme-dioxygen complex, that is best described as a ferric superoxy species \([\text{Fe(III)}−\text{O}_2^{-}]\) (1,20-23). This intermediate does not react with L-arginine but may (24) or may not (25) react with NOHA. Addition of oxygen to reduced eNOS forms two distinct heme-oxy species, which have been interpreted as the ferrous-dioxygen complex and the ferric-superoxy complex (26).

H\textsubscript{\text{\textomega}}B acts as an electron donor to the ferric-superoxy species in both steps of NO synthesis (Fig. 1). In the first step the reductase domain reduces the H\textsubscript{\text{\textomega}}B•\textsuperscript{\text{\textomega}} radical (18,27-33). In the second step, a downstream reaction intermediate (25), possibly a ferrous-heme NO complex (34), reduces the H\textsubscript{\text{\textomega}}B•\textsuperscript{\text{\textomega}} radical. Reduction of the Fe(II)−O\textsubscript{2}•\textsuperscript{\text{\textomega}} species at cryogenic temperatures results in a ferric heme peroxy species that rapidly reacts at higher temperatures with either L-arginine or NOHA to form products (35). Unlike, heme-oxygenases such as cytochrome P-450 or heme oxygenase (36,37), a ferric heme-hydroperoxo species has not been observed in cryo annealing experiments.

Bacterial NOSs retain NO in their heme pockets for longer times compared to their mammalian counterparts (38). In *Bacillus subtilis* NOS (bsNOS), the release of NO is 20 fold slower than that in mammalian NOSs due to a bacterially conserved Val to Ile switch, which offers more steric hindrance for the heme-bound NO to diffuse away from the heme. An Ile to Val mutation in bsNOS increases the rate of NO release 3.6 times and a Val to Ile mutation in mouse iNOS increases the rate of NO release by 3 times (38). Thus, the NOS heme pocket can tune the reactivity of heme bound species.

Otherwise unstable reaction intermediates of cytochromes P-450, another class of well-studied heme-containing monooxygenases, have been observed at cryogenic temperatures, after radiolytic reduction of the heme (39-41). Additionally, in cysteine-ligated heme proteins, such as P450cam and chloroperoxidase, rapid formation of Compound I (Cpd I) (Fe\textsuperscript{\text{\textomega}}=O centered radical) and related species can be achieved on reaction with peracids (42-45). In these cases, Cpd I (absorption peak ~367 nm) forms in ~10 ms after rapidly mixing 3-chloroperbenzoic acid with the ferric enzyme. Within 40 ms, this species converts to an inactive product with a peak ~406 nm (42).

In NOS, the reactive heme-oxygen species that react with L-arginine or NOHA have not been observed, though it is widely thought that the Cpd I species is involved, in analogy to cytochrome P450-type reactions (43,44,46). Work with cytochrome P450 homologues (44) has shown that thermophilic prokaryotes can be exploited as a source of thermostable enzymes that have slow reaction profiles at temperatures below 25 °C. With this motivation, we have cloned, expressed and characterized a thermophilic nitric oxide synthase like protein from *Geobacillus stearothermophilus* (gsNOS). Herein, we characterize biophysical and biochemical properties of gsNOS and demonstrate its enhanced thermostability and slower reaction kinetics compared to other bacterial NOSs. We also show that the N-terminal extension contributes to this thermal stability. The most striking property of gsNOS is that it forms a stable heme-oxygen complex that persists on the timescale of seconds, at 4 °C. The crystal structure of gsNOS at 3.2 Å resolution reveals very high similarity to the structure of bsNOS and provides insight into the slower reactivity of gsNOS.
EXPERIMENTAL PROCEDURES

Materials and methods. Dioxane and sodium chloride were obtained from Mallinckrodt, (+)-2-methyl-2,4-pentane diol (MPD) from Hampton Research and Tris (hydroxymethyl) aminomethane from Fisher Scientific. All other chemicals were obtained from Sigma-Aldrich, unless otherwise noted. All UV-Visible spectra and kinetic data were recorded using an Agilent 8453 UV-Visible Spectroscopy system. SVD analysis was done using the program SPECFIT (47–50).

Molecular Biology. The NOS gene of G. Stearothermophilus (ATCC strain number 12980) was amplified by PCR from genomic DNA. The 5′ primer generated an NdeI site before the start codon and the 3′ primer generated an XhoI site after the stop codon. The amplified fragment was cloned into the pET28 expression vector (Novagen) and transformed into E. coli BL21(DE3) cells.

Protein Expression and Purification. The full length NOS (gsNOS) and a shorter construct with the first 13 residues removed from the N-terminus (gsNOS+13) were overexpressed in E. coli BL21(DE3) cells with a 6-His tag. The proteins were purified using Ni-chelate chromatography and then size-exclusion chromatography, after removal of the 6-His tag with thrombin. Both the constructs could be concentrated to ~100mg/ml, as estimated by the Bradford Assay.

Crystallization. GsNOS produced orthorhombic crystals of dimensions 200-400 µm in 24-48 h at 22°C when grown by vapor diffusion from 45-50 mg/ml protein in 50mM Tris (pH 7.5), 150mM NaCl, mixed with freshly dissolved 1-2 mM L-arginine and 1-2 mM L-tryptophan (Trp). Trp was added to help stabilize the pterin-binding site due to evidence that it will bind there in D. radiodurans NOS (16). The reservoir was mixed 1:1 with protein solution and contained 40-46% dioxane and 10% MPD. GsNOS crystals were of space group P2₁2₁2 with cell dimensions 154.0 Å x 118.7 Å x 49.8 Å. The crystals contained two NOS subunits per asymmetric unit.

Structure determination. Diffraction data were collected at 100 K with synchrotron radiation (λ = 1.002 Å) on beamline X-25 of the National Synchrotron Light Source (NSLS) at BNL. The data sets were reduced and scaled using HKL2000 (51). Initial phases were determined by molecular replacement (AmoRe) (52) with the structure of NOS from B. Subtilis as the probe (PDB entry 1M7V). The model was then refined in CNS (53) using standard positional and thermal factor refinement and the structure adjusted with XFIT to Fobs – Fcalc and 2Fobs – Fcalc maps. Addition of L-arginine, heme and water molecules amidst cycles of refinement produced the final model.

Nitrite Formation with Peroxide. 30-50 µM enzyme was incubated at different temperatures with 1 mM L-arginine and 20 mM H₂O₂ and the reaction was stopped at different times by adding Griess reagents R₁ (Sulfanilamide) and R₂ (N-(1-Naphthyl) ethylenediamine). The product (pink dye) formation was monitored by measuring the absorbance at 540 nm. The amount of nitrite generated in the solution was calculated using the Griess assay kit from Cayman Chemicals. Each activity reported represents an average from at least three experiments.

Single Turnover Experiments. Concentrated full-length gsNOS was cycled through a degassing chamber and left in an anaerobic glove box for ~30 min. All buffer solutions were extensively degassed and placed under argon. Degassed buffer solution was used to dilute the protein solution suitably for UV-Vis spectroscopy. To observe the spectral changes the enzyme undergoes during reaction with oxygen, gsNOS (13 µM) was reduced by titration with sodium dithionite (30-50 µM). The cuvette containing reduced gsNOS (13 µM) was sealed using a rubber septum inside the glove box and then transferred to a UV-Vis spectrophotometer.
The temperature of the cell with the sample in the UV-Vis spectrophotometer was lowered to 4 °C. Nitrogen gas was blown around the cuvette to prevent water condensation due to lowered temperature. Ice-cold air saturated buffer was injected into the cuvette through the rubber septum to start the reaction, at which point the sample contained ~8 µM GsNOS, ~20-30 µM dithionite and ~160 µM oxygen (54). The solutions were mixed rapidly using a magnetic stir-bar in the cuvette.

RESULTS

General properties. GsNOS was PCR cloned and expressed with a His<sub>6</sub> affinity tag in E. coli. The protein has 65% sequence identity when compared to the NOS protein from the related mesophile B. subtilis (Supplemental Fig. 1). After nickel-NTA affinity purification and proteolytic cleavage of the His-tag, gsNOS (~43 KDa / subunit), elutes on a gel filtration column as a dimer (apparent MW ~86 KDa). The UV-Vis spectra of free gsNOS (absorption maxima at 403 nm and 519 nm), imidazole bound enzyme (427 nm and 553 nm), L-arginine bound enzyme (399 nm and 517 nm), and reduced enzyme with L-arginine bound (415 nm and 552 nm) (Fig. 2) are very similar to those of mammalian and other bacterial NOS oxygenase domains (1,11,12,20-22). Shifts in the Soret peak indicate that the heme coordinates imidazole (427 nm), and can be displaced by L-arginine (Fig. 2).

Thermal stability. GsNOS shows high thermal stability. Molar ellipticity measurements (Θ<sub>222</sub>) of NOS dimers indicate irreversible loss of secondary structure with increasing temperature (Fig. 3). We define the melting temperature as that at which half of the ellipticity (Θ<sub>222</sub>) is lost as temperature is raised. Whereas bsNOS melts at 60 °C, full-length gsNOS melts at 80 °C. GsNOS+13, in which an N-terminal amino acid extension has been removed, melts at an intermediate temperature of 66 °C (Fig. 3). In all cases, loss of secondary structure, as evidenced by CD, is irreversible.

Activity. Activity of gsNOS and bsNOS were compared at different temperatures by evaluating the rate of nitrite produced from substrate L-arginine in the presence of hydrogen peroxide (Table 1). The amount of nitrite formed was quantitated by the Griess reaction (11) at 25 °C after incubation of the L-arginine saturated enzyme with peroxide at various temperatures and times. Product formation was linear with time. We followed the conversion of L-arginine rather than NOHA to NO, because NOHA breakdown at elevated temperatures resulted in significant background product formation. At increased temperatures, gsNOS gives a lower rate of product formation than bsNOS. At 50 °C, the bsNOS activity deviates greatly from that of gsNOS, which increases gradually with temperature. At temperatures above 50 °C, bsNOS denatures and precipitates. GsNOS reactions with peroxide and NOHA instead of L-arginine, give ~2 times more nitrite formation at room temperature.

Single Turnover Experiments.

Substrate free gsNOS. In the absence of any substrate, the spectrum recorded ~ 5 sec after introduction of oxygen showed a red-shifted Soret peak at 413 nm (Fig. 4) and the line shape was considerably different to those of mammalian and other bacterial NOS oxygenase domains (1,11,12,20-22). Shifts in the Soret peak indicate that the heme coordinates imidazole (427 nm), and can be displaced by L-arginine (Fig. 2).
contribution from free ferric enzyme. Nevertheless, combination of spectra from the substrate-free ferric and ferrous forms cannot explain the optical features of the intermediate observed in the presence of oxygen. When the same reaction was carried out in the presence of 40 µM H$_2$B, which is known to accelerate the decay of the ferrous-oxy species in mammalian and bacterial NOSs (21,32), the conversion of the 413 species to the ferric form was complete in ~ 40 s at 4 °C. With 100 µM H$_2$B, the intermediate decayed to the Fe(III) form within 5 s and it could not be observed by these methods. In presence of H$_2$B, an oxidized form of H$_2$B that binds NOS and is redox inactive, the reaction mixture behaved very similarly to the reaction in the absence of pterin. Complete conversion to ferric form was again possible only after heating the cell to 25 °C.

L-Arginine bound gsNOS. The foresaid reactions were repeated in the presence of 50 mM L-arginine. The ferric form of gsNOS with L-arginine bound has a Soret peak at 399 nm, whereas dithionite-reduced gsNOS had a Soret peak at 415 nm (Fig. 1). After oxygen injection, the first spectrum taken at t = 5 sec showed a Soret peak at 427 nm, which decayed completely to 399 nm in ~ 1 min (Fig. 5). Four isosbestic points in the spectra indicate a smooth transition from the 427 nm species to the ferric heme species. Rate constants derived from loss of the 427 nm peak or gain of the 399 nm peak were identical within error. This behavior resembles the ferrous-oxy complex of mammalian NOSs at ~30°C (21,22,26) or at ambient temperatures using stop-flow techniques (20). A 428 nm band is also characteristic of a ferrous-heme-oxy complex in chloroperoxidase (55).

H$_2$B accelerates the decay of the ferrous-oxy species and appearance of the ferric heme species (Fig. 6). In the presence of 15 µM H$_2$B, the decay rate of the 427 nm band and the appearance rate of the 399 nm were both biphasic, each with a dominant phase that was much faster than that observed in the absence of H$_2$B (Table 2). Increasing the concentration of H$_2$B to 40 µM did not noticeably affect the relative proportions the slow and the fast phase. Furthermore, rate constants for the slow phases are not same as those observed in the absence of pterin (Table 2). Because of these considerations, and a binding constant of H$_2$B to B. subtilis NOS (100 nM) (12) that is much lower than the concentrations being used here, the slow phase in the presence of H$_2$B is unlikely to derive from gsNOS free of H$_2$B.

With H$_2$B, the fast phases were more difficult to characterize from 399 nm peak appearance rate than from 427 nm peak disappearance due to apparent smaller amplitudes (Table 2). This may indicate that the kinetics of the optical changes at these two wavelengths are not same when H$_2$B is present. Such behavior could result from the presence of another intermediate between the ferrous-oxy and Fe(III) states of gsNOS with H$_2$B. Better time resolution is necessary to address whether this is truly the case.

In contrast, 15 µM H$_2$B had a more minor, but opposite effect on the rates for Fe(III) formation or Fe(II)-O$_2$ complex decay (Table 2). Rates derived from 427 nm and 399 nm were again equivalent and monophasic, but slightly less than with free enzyme, suggesting a modest stabilization of the ferrous-oxy species by H$_2$B.

N$^\omega$-hydroxy L-arginine bound gsNOS
The same reactions were repeated under identical conditions with NOHA (1 mM) as the substrate, and spectra recorded in 0.5 sec intervals after the addition of air-saturated buffer. The spectral characteristics of the oxidized and reduced form of gsNOS with NOHA were similar as those observed with L-arginine, with the Soret bands at 399 nm and 415 nm, respectively. In the absence of pterin, no intermediates are observed and the Fe(III) form of the enzyme slowly reappears, within 1 min. However, in the presence of 60 µM H$_2$B, the first spectrum recorded 0.5 s after the addition of air-saturated buffer had 3 major peaks at 399 nm, 423 nm and 440 nm (Fig. 7A). These three peaks correspond to the Soret absorption bands of Fe(III) gsNOS, and
the mammalian NOS Fe(II)-O₂ complex in the presence NOHA (20-22,26) and the mammalian NOS Fe(III)-NO product complex (1,25,56), respectively. In comparison to mammalian NOS enzymes, this reaction should form a Fe(II)-O₂ intermediate followed by a Fe(III)-heme nitrosyl complex. Single-value decomposition (SVD) of the transient absorption spectra taken with 0.5 s time resolution could not unambiguously resolve a ferrous-oxy intermediate from a ferric-NO intermediate. However, SVD did resolve two principle components to the spectral transition: 1) a product Fe(III)-heme spectra and 2) an “intermediate” with spectra features characteristic of a mixture of an Fe(III)-NO complex, with another species, probably the Fe(II)-O₂ species, due to the 423 nm absorption maximum (Fig 7B). Notably, this spectrum only occurs in the presence of both NOHA and H₂B. The decay rate of the 440 nm signal (~0.04 s⁻¹), representative of Fe(III)-NO, matches well the recovery of the 399 nm peak, representative of Fe(III) (Table 3), as well as the SVD-derived rate constant for conversion of the “mixed” intermediate to product Fe(III) heme (0.04 s⁻¹). Simulation of the kinetics for the sequential conversion of heme-oxy to Fe(III)-NO to Fe(III) indicates that the heme-oxy and Fe(III)-NO could appear to decay with the same observed rate constant during the period of observation if the rate constant for the Fe(III)-NO decay is ~2 times that for the conversion of heme-oxy to Fe(III)-NO. Similar to the Fe(III)-O₂ complex, the Fe(III)-NO in gsNOS appears to be much more stable (5-50 times) than in other NOSs (Table 3).

Crystal Structure

At 3.2 Å resolution (Table 4), the overall structure of gsNOS (Fig. 8A) appears strikingly similar to the structure of NOS from B. subtilis (bsNOS) (15), except for a few significant changes near the heme that result in a more compact active site. GsNOS maintains the overall fold of mammalian NOSoxy enzymes, with a conserved β-winged core surrounded by α-helices (57). As with other bacterial NOS proteins, the N-terminal hook, the pterin binding segment and the zinc-binding site are absent. The dimer interface in gsNOS is identical to the bsNOS dimer interface, with the exception of a change from Ala334 to Thr343 (gsNOS) in the helical lariat region (15). The side-chain of Thr343 hydrogen bonds with the peptide carbonyl of a conserved Ser340 (gsNOS) of the same monomer, but does not alter the backbone conformation. A long loop in bsNOS consisting of 10 residues joining two β-strands (Pro110 to Val119) is replaced in gsNOS by a shorter loop consisting of only six residues (Arg124 to Val129). The overall structure of gsNOS appears somewhat more compact than the structure of bsNOS (Fig. 8B). The lower part of the dimer comes closer and the upper part moves apart. The dimer interface, however, overlaps nearly exactly.

Perhaps, the most significant difference in the structure of gsNOS is in the active site (Fig. 9). A Lys356 (bsNOS) to Arg365 (gsNOS) substitution results in a hydrogen bonding interaction between this residue and Asp225 in gsNOS (Asp216 in bsNOS) that alters the orientation of the Asp carboxylate relative to bsNOS. Reorientation of Asp225 displaces Ser224 and pushes Ile223 about ~0.6 Å closer towards the heme iron in gsNOS (Fig. 9). Although the limited resolution of the gsNOS structure (3.2 Å) prevents a precise evaluation of the Ile223 side chain position, Difference Fourier electron density maps clearly show that Ile223, and its β-strand, reside closer to the heme than in bsNOS. Surprisingly, there is no apparent electron density for the N-terminal extension that confers enhanced thermal stability to gsNOS. SDS-PAGE analysis of crystals showed that the N-terminus was not proteolytically removed from the crystallized protein.

DISCUSSION

We have cloned and characterized a NOS-like protein from G. stearothermophilus that is very similar to all other well-characterized NOSs, in terms of its sequence (Supplemental Fig. 1), structure, substrate specificity, activities and spectral properties. We demonstrate that gsNOS produces nitrite from L-arginine and NOHA in the peroxide
shunt reaction and an optical signal characteristic of \textit{bona fide} NO in single turnover experiments when only NOHA and H$_2$B are present. The structure of bsNOS reveals absence of the zinc site and N-terminal hook of mammalian NOSoxy proteins and thus, this protein further demonstrates that these regions are not required for catalysis provided the dimer remains intact (11,12,58). GsNOS has a small N-terminal extension (~10 residues) compared to NOSs from other bacteria like \textit{D. radiodurans}, \textit{B. subtilis} and \textit{S. aureus}. For all thermophiles for which genome sequences are available, only one other, \textit{Geobacillus kaustophilus} also has a NOS gene. GkNOS shares 94\% sequence identity with gsNOS, but has a much longer N-terminal extension (~50 residues). \textit{G. kaustophilus} has genes coding for enzymes that synthesize H$_4$B, and the \textit{G. stearothermophilus} genome (currently incomplete) contains homologs with ~90\% sequence identity.

Unlike other NOSs, gsNOS has high thermal stability, with a melting temperature of 80 °C, 20 °C higher than the melting temperature of bsNOS. Interestingly, removal of 13 residues that extend the gsNOS protein beyond bsNOS reduces the melting temperature to only 6 °C above that of bsNOS. Surprisingly, these residues are completely disordered in the crystal structure. The gsNOS rate of catalysis is much slower compared to other bacterial NOSs and mammalian NOSoxy domains at ambient temperatures, consistent with enzyme operating above 60 °C \textit{in vivo}. This sluggish reactivity has allowed us to carry out single turnover experiments that reveal a slow rate of conversion to products and uncharacteristically stable heme-oxy intermediates.

Oxygen complexes of reduced NOSs, cytochrome P450s, and chloroperoxidases are well characterized (1,20-23,36,39,41,59-64). In P450cam, multiple, on pathway heme-oxy complexes can be resolved by stopped flow methods (65-68). Furthermore, studies with peroxo acids as oxo donors have visualized Cpd I and related ferryl intermediates (42-45,60,69). For eNOS, recent work has described two distinct oxygen complexes, which differed in their reactivity towards CO at -30 °C (26). The complex which forms in the absence of substrate and exchanges O$_2$ with CO is thought to be the ferrous-oxygen complex [Fe(II)-O$_2$] (420 nm, 560 nm), whereas, the complex which readily forms in the presence of substrate and does not exchange O$_2$ with CO is thought to be the ferric-superoxy complex (432 nm, 564 nm). Although, the Fe(II)-O$_2$ and Fe(III)-O$_2^-$ species can be formally considered resonance structures, in any given case, the electronic structure of the heme-oxy complex may favor an O-O bond more indicative of one form than the other (1).

Single turnover experiments with gsNOS show that in the absence of any substrate, after the addition of oxygen, a stable spectral state forms that is spectroscopically distinct from the ferrie or ferrous forms of the enzyme. 40 μM H$_2$B accelerates the rate of decay of this species to the ferric form, whereas H$_2$B, a redox inactive pterin does not have any significant effect on the intermediate species observed. These observations indicate that the intermediate formed in the absence of L-arginine (413 nm) is an oxygenated heme complex. In the presence of L-arginine, the Soret peak of the intermediate shifts to 427 nm and again H$_2$B accelerates the decay of this species to the ferric enzyme. This is a hallmark for H$_2$B acting to reduce the Fe(III)-O$_2$ complex. However, the kinetics are not as straightforward to characterize because the decay rates are much faster in the presence than in the absence of H$_2$B. Rates for gain of the Fe(III) (399 nm) and loss of Fe(III)-O$_2^-$ (427 nm) may not be the same when H$_2$B is bound (Table 2). This may indicate additional observable spectral intermediates may manifest between the Fe(III)-O$_2^-$ and Fe(III) states when both L-arginine and H$_2$B are present.

In general, heme-oxy complexes of thiolate-ligated hemes have Soret bands that range from 414 nm to 432 nm (1,11,12,20-22,26,41,59) (Table 5). These spectral differences have been correlated with the changes in hydrogen bonding to the proximal heme thiolate which blue-shifts the Soret peak,
and hydrogen bonding to the proximal oxy-species, which red shifts the Soret peak (46,70,71). The studies of eNOS mentioned above correlate well with this general trend. In nNOS, substrate L-arginine or NOHA binds closely to the site of O₂ heme coordination and participates in an H₂O–guanidinium hydrogen-bonding network with the coordinated O₂ (35,57,58,72). Thus, interactions of the substrate are expected to increase hydrogen bonding to the coordinated oxygen and red shift the Soret. In accord with increased hydrogen bonding, the Soret spectra of the heme-oxo complex in eNOS red shifts from 420 nm to 432 nm in the presence of L-arginine and 428 nm in the presence of NOHA. In nNOS, the heme-oxo complex has a Soret peak at 427 nm in the presence of L-arginine (20,73), a 416 nm peak in the absence of L-arginine (21) and a 419 nm peak in the presence of inhibitor N⁸-methyl L-arginine, which cannot form a hydrogen bond with heme-bound oxygen (Table 5). Similar trends are seen in gsNOS at 4 °C, except that the substrate free form (413 nm) and the substrate bound form (427 nm) have slightly blue shifted Soret bands relative to those of eNOS and similar to those of nNOS. In nNOS, the 430 nm heme-oxo species has been definitively assigned as a ferric-superoxy species by resonance Raman Spectroscopy (1,74). Thus, increased hydrogen bonding to the distal O₂ oxygen induced by substrate will favor Fe(III)-O₂⁻ over Fe(II)-O₂ in NOS.

In presence of NOHA and absence of HaB, no striking spectral changes are observed after addition of oxygen and conversion to the ferric form of the enzyme follows. Addition of H₂B yields a small stabilization of the heme-oxo complex, but does not generate new spectral species we can observe. However, in the presence of 60 μM H₂B, the spectrum immediately after oxygen addition shows an overlap of three distinctive Soret peaks. The system returns to an Fe(III)-heme state (399 nm) through an intermediate spectra containing major absorption peaks consistent with a mixture of a Fe(III)-NO species (440 nm) (1,25,56) an a heme-oxygen complex in the presence of NOHA (423 nm) (20-22,26). Anaerobic stopped-flow methods will be better able to define the rate constants and progression of these intermediates, but this data does provide strong evidence for formation of an Fe(III)-NO after reaction of heme-bound oxygen with NOHA in the presence of H₂B. As no Fe(III)-NO complex forms with H₂B, this data also corroborates H₂B acting as an electron donor in the second step of the NOS mechanism (18,25,75).

Rapid-freeze EPR experiments on the mouse iNOS oxygenase domain show that a tetrahydrobiopterin radical forms and then becomes reduced during NOHA oxidation, resulting in Fe(III)-NO formation (25). In contrast to the mammalian NOSs, and other bacterial NOSs, where the ferric-superoxy appears on milli-second timescales at 10 °C or at extremely low temperatures (~30°C) (20-22,26), the rate of decay of the ferric-superoxy species in gsNOS in ~10-100 times slower. Similarly, the Fe(III)-NO species in gsNOS in 5 times more stable than in bsNOS, and 50 times more stable than in iNOS (Table 3).

The crystal structure of gsNOS provides some insight into why the heme-ligand complexes survive 1-2 orders of magnitude longer than counterparts do in other bacterial NOSs and mammalian NOSoxy (Table 2). Given the different behaviors of the mesostable bsNOS and the thermostable gsNOS, there are very few structural differences between the two enzymes. The stability of the heme-oxo complex in gsNOS could derive from a number of factors that include, a decreased heme redox potential (76), increased hydrogen bonding to the heme ligand, or affects on steric and dynamic properties of the heme pocket. Changes to heme redox potential or heme-ligand hydrogen bonding are unlikely as there are no differences in charged residues or hydrogen bond donors in the heme pockets of gsNOS compared to the bsNOS. However, there is a conformational difference in gsNOS at a key residue (Ile233) known to generally affect NOS heme-ligand dissociation rates.

A conserved valine residue in mammalian NOS that resides above the heme pocket switches to a conserved isoleucine in
bacterial NOSs (Supplemental Fig. 1). This Ile has been shown to reduce the release rates of heme-bound NO (38). Comparison of the respective crystal structures show that the same Ile223 (present in both bsNOS and gsNOS) is ~0.6 Å closer to the heme in gsNOS than in bsNOS. This appears to be linked to the substitution of Arg365 (gsNOS) for Lys356 (bsNOS) (Fig. 9). A more constrained heme pocket in gsNOS may disfavor ligand dissociation. Consistent with proximity of the residue at position 223 to the heme iron decreasing ligand dissociation rates, the Fe(III)-NO species that forms after reaction with NOHA, resides on the heme even longer in gsNOS than compared to bsNOS (Table 3). Sequence alignment with all other known bacterial NOSs and mammalian NOSoxy domains (data not shown) shows that only gsNOS and the other thermophilic *G. kaustophilus* NOS have arginine at this key position.

Recent structures of cytochromes P-450 bound to oxygen show that the water molecule structure in the active center changes when oxygen ligates the heme (61,63,64). Similarly, water molecules not present in the ligand free structures of gsNOS may affect the stability of the heme-oxy species in bacterial NOS. Even small motions in Ile233 could in turn influence hydrogen bonding patterns between solvent and heme ligand and thereby contribute to differences in ferrous oxy stability.

There is no obvious single contributing factor to the overall thermal stability of gsNOS, although the structure does appear more compact than that of bsNOS (Fig. 8B). Surprisingly, removal of N-terminal extension (~13 residues) decreases the Tm almost to that of bsNOS; yet, this region is completely disordered in the crystal structure. We have previously observed that the stability and solubility of bacterial NOSs depend highly on the N-terminus (15). Perhaps removal of the extension is sufficiently destabilizing to overcome small structural features that collectively generate increased stability in gsNOS.

**CONCLUSIONS**

We have characterized a thermostable nitric oxide synthase, with a slower reaction profile. As a result, the heme-bound oxygen intermediates can be observed at ambient temperatures on the timescale of seconds. Dramatic shifts in the heme-oxy spectra correlate with substrate hydrogen bonding to heme-coordinated O2. This interaction likely converts a heme-oxy complex from an electronic state best described as ferrous-oxy (413 nm), to one better represented as ferric-superoxy (427 nm). Structural comparisons suggest that subtle rearrangements in the distal heme pocket contribute to increased stability of heme-oxy complexes in gsNOS. The slow catalytic profile of the enzyme may be an advantage for identifying the nature of the reactive heme-oxygen intermediates directly involved in the NO-synthase mechanism.
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FOOTNOTES

The abbreviations used are: NOS, Nitric Oxide Synthase; NO, Nitric Oxide; FAD, Flavin Adenine Dinucleotide; FMN, Flavin Mono Nucleotide; NADPH, Nicotinamide Adenine Dinucleotide Phosphate (reduced form); H4B, (6R)-tetrahydro-L-biopterin; H2B, Dihydro-L-biopterin; Cpd I, [Fe⁴⁺-O centered radical] Compound I; NOHA, Nω-hydroxy L-arginine; PCR, Polymerase Chain Reaction; GkNOS, Geobacillus kaustophilus NOS; DeiNOS, D. Radiodurans NOS.

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The atomic coordinates and structure factors (code 2FLQ) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

FIGURE LEGENDS

FIG. 1. Current mechanistic model for NO biosynthesis by NOS adapted from reference (18). Formation of citrulline, NO and ferric-heme marks the end of one catalytic cycle. In some mammalian NOS isozymes, further reduction of the ferric-heme nitrosyl complex competes with NO release from the active center (18).

FIG. 2. UV-Visible spectra of gsNOS under various conditions. The Soret peak of substrate free gsNOS, 403 nm, shifts to 427 nm on the addition of imidazole (5mM), and then shifts to 399 nm on the addition of L-arginine (1 mM). Reduction of this enzyme with sodium dithionite results in a Soret maximum of 415 nm. Inset: Enlarged visible absorption spectra.

FIG. 3. Temperature melting curves for gsNOS (—), gsNOS+13 (- - -) and bsNOS (-----).

FIG. 4. UV-Vis spectra of reduced substrate free gsNOS (Absorption maximum 411 nm), the intermediate formed after addition of oxygen to substrate free gsNOS (Absorption maximum at 413 nm), re-oxidized ferric gsNOS (Absorption maximum at 403 nm).

FIG. 5. UV-Vis spectra of the gsNOS heme-oxygen complex (Absorption maximum at 427 nm) converting to the ferric form (Absorption maximum at 399 nm). Spectra were taken every 5 sec.
FIG. 6. H₄B but not H₂B accelerates decay of the ferrous-oxy complex. Rates of decrease in absorbance at 427 nm, which corresponds to the absorption maximum of the heme-oxygen complex, in presence of substrate L-arginine (R), R + H₂B and R + H₄B. Rates of absorption increase at 399 nm have similar kinetics.

FIG. 7. GsNOS converts NOHA to a ferric-NO species in the presence of H₄B. UV-Vis spectra taken in 5 s second intervals after addition of air-saturated buffer in the presence of 1 mM NOHA and 60 µM H₄B. (A) The first spectrum taken 0.5 s after addition of oxygen has 3 peaks, at 399 nm, 423 nm, and 440 nm, which correspond to Soret maxima for the ferric enzyme, heme-oxygen complex and a Fe(III)-NO complex. The enzyme finally returns to the ferric form. The spectra shown were recorded at 5 sec intervals. (B) Singular Value Decomposition resolves two principle contributions to the transient spectra as shown: (1) Fe(III)-heme spectrum (399 nm) and (2) An “intermediate” spectrum with features characteristic of an Fe(III)-NO complex (440 nm) and probably the ferric-superoxy species (423 nm).

FIG. 8. Overall structure of gsNOS. (A) The subunits (blue and pink ribbons) form a tight dimer characteristic of all NOSoxy domains. Heme groups (yellow bonds) lie at the bottom of a channel that opens in the center front of the blue subunit. (B) Superposition of the Cα traces of gsNOS (blue) and bsNOS (dark orange). The topologies of the proteins are highly similar, although gsNOS appears slightly more compact.

FIG. 9. Comparison of the active sites of bsNOS (orange) and gsNOS (yellow). In bsNOS, Lys356 does not interact with Asp216. A Lys to Arg substitution in gsNOS allows Arg365 to hydrogen bond with Asp225 (3.2 Å), altering its side chain position. This change in structure appears to be correlated with movement of Ser224 that in turn pushes Ile223 into the active site, reducing the distance between δ-carbon of Ile223 and the heme iron atom from 6.7 Å (bsNOS) to 6.1 Å (gsNOS).
### Table 1. Rates of nitrite production by NOSs from various sources as a function of temperature.

| Temperature | [NO₂⁻] production gsNOS (min⁻¹ heme⁻¹) (k_{cat} × 100) | [NO₂⁻] production bsNOS (min⁻¹ heme⁻¹) (k_{cat} × 100) | [NO₂⁻] production deiNOSa (min⁻¹ heme⁻¹) (k_{cat} × 100) | [NO₂⁻] + [NO₃⁻] production nNOSb (min⁻¹ heme⁻¹) (k_{cat} × 100) |
|-------------|--------------------------------------------------------|--------------------------------------------------------|--------------------------------------------------------|--------------------------------------------------------|
| 25 °C       | 2.6 ± 0.1                                              | 4.6 ± 0.1                                              | 7.5 ± 0.5                                              | 28 ± 3                                                 |
| 35 °C       | 3.6 ± 0.2                                              | 13.3 ± 0.3                                             | --                                                    | --                                                    |
| 50 °C       | 10.3 ± 0.1                                             | 50.3 ± 1.1                                             | --                                                    | --                                                    |

a Reference (16)  
b Reference (27)

### Table 2. Kinetic parameters for single turnover experiments. Rates of decay of the heme-oxygen complex (as monitored at 427 nm) and the appearance of ferric heme enzyme (399 nm) in the presence of 2.5 mM L-arginine, compared to the rates of other NOSs for which similar rates are known. Reduced pterin, H₂B (15µM) accelerates both the decay of the heme-oxygen complex and the ferric heme appearance. Each kinetic parameter represents an average from at least three separate experiments. The relative amplitude of each phase is given in bracket for bi-exponential kinetics.

| System                  | Fe(III) formation rate (s⁻¹) (k x 1000) | Fe(H)-Oxygen decay (s⁻¹) (k x 1000) | Fe(II)-Oxygen decaya (s⁻¹) (k x 1000) | Fe(II)-Oxygen decayb (s⁻¹) (k x 1000) | Fe(II)-Oxygen decayb (s⁻¹) (k x 1000) |
|-------------------------|----------------------------------------|------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|
| NOS + Arg               | 40 ± 3                                  | 47 ± 5                             | 1370 ± 40                            | 400 ± 200                            | 140                                  |
| NOS + Arg + H₂B         | 24 ± 3                                  | 28 ± 3                             | 1400 ± 150                           | 76 ± 21                              | 10000                                |
| NOS + Arg + H₂B         | k₁ = 400 (f=N.D.)                        | k₂ = 8 ± 2 (f=0.15)                | k₁ = 10000 (f=0.85)                  | k₂ = 76 ± 21 (f=0.15)                | 3900 ± 200                           |

a Reference (11)  
b Reference (12)  
c Reference (20)  
d N.D. not determined due to inaccuracy caused by the rapid decay of the fast component.
Table 3. Kinetic parameters for single turnover experiments with NOS, NOHA and H₄B

| Transformation                      | gsNOS (4 °C) | bsNOS (10 °C) | iNOS (10 °C) |
|-------------------------------------|--------------|---------------|--------------|
| Fe(III) formation rate              | 40 – 80      | 210 ± 5       | 2300 ± 100   |
| (k x 1000) (s⁻¹)                    |              |               |              |
| Fe(III)-NO decay                    | 50 – 100     | 210 ± 5       | 2300 ± 100   |
| (k x 1000) (s⁻¹)                    |              |               |              |

a Reference (12)
b Reference (25)

Table 4. Data collection and Refinement Statistics for gsNOS complexed with L-arginine.

| Parameter                              | Value          |
|----------------------------------------|----------------|
| no. of residues                        | 717            |
| ligand                                 | L-arginine     |
| number of waters                       | 223            |
| resolution (Å)                         | 3.2 (3.20 – 3.29) |
| number of unique reflections           | 15805          |
| number of observations                 | 60395          |
| % completeness                         | 92.0           |
| (I/σI)a                                | 12.9 (5.5)     |
| R_Sym b (%)                            | 17.0 (48.3)    |
| R¹ (%)                                 | 22.2 (31.3)    |
| R_free d (%)                           | 29.5 (36.2)    |
| overall ⟨B⟩g (Å²)                      | 21.2           |
| mainchain ⟨B⟩ (Å²)                     | 18.4           |
| sidechain ⟨B⟩ (Å²)                     | 24.4           |
| rmsd for bondsf (Å)                    | 0.0083         |
| rmsd for anglesf (degrees)             | 1.511          |

a Intensity of the signal to noise ratio. b R_Sym = ΣΣ|I_j|−⟨|I|⟩|ΣΣ|I_j|. c R = Σ|F_obs|−|F_calc|/Σ|F_obs| for all reflections (no σ cutoff). d R_free calculated against 10% of reflections removed at random. e Overall model average thermal ⟨B⟩. f Root mean square deviations from bond and angle restraints. g Highest resolution bin for compiling statistics.
Table 5. Soret peak position of several thiolate-ligated heme proteins in presence and absence of substrates. Soret peaks are red-shifted relative to free enzyme when substrate can hydrogen bond with heme-bound oxygen. In the case of CytP450cam (P. putida) and bacterial CytP450, the 418 nm oxy-species can exchange with CO, like the 420 nm species in substrate-free eNOS.

| Experiment                      | Temperature (°C) | Soret peak (nm) | Reference |
|---------------------------------|------------------|-----------------|-----------|
| nNOSoxy + Arg                   | 25               | 430             | (74)      |
| nNOSoxy + Arg                   | 10               | 427             | (20)      |
| nNOSoxy + N^6^-methyl Arg       | -30              | 419             | (22)      |
| nNOS + Arg                      | 10               | 427             | (73)      |
| nNOS                            | -30              | 416             | (21)      |
| iNOSoxy + Arg                   | 10               | 427             | (30)      |
| eNOS                            | 7                | 420             | (26)      |
| eNOS + Arg                      | 7                | 432             | (26)      |
| eNOS + NOHA                     | 7                | 428             | (26)      |
| P450 (liver microsomal)         | 25               | 418             | (77,78)   |
| P450_{SCC}                      | -30              | 422             | (79)      |
| P450_{SCC}                      | -17              | 420             | (80)      |
| P450_{SCC} + Cholesterol        | -17              | 423             | (80)      |
| CytP450cam (P. putida)          | 4                | 418             | (81,82)   |
| CytP450 (bacterial)             | -30              | 418             | (83)      |
| Chloroperoxidase                | 25               | 430             | (84)      |
| Chloroperoxidase                | 25               | 428             | (55)      |
| gsNOS                           | 4                | 413             | This work |
| gsNOS + L-arginine              | 4                | 427             | This work |
| gsNOS + NOHA                    | 4                | 423             | This work |
FIGURES

Figure 1
Figure 2
Figure 4
Figure 5
Figure 6

[Graph showing the absorbance changes over time for H4B + Arginine and H2B + Arginine]
Figure (7B)
Figure 8

A

B
Structure and reactivity of a thermostable prokaryotic nitric oxide synthase that forms a long-lived oxy-heme complex
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