Nitric-oxide synthases (NOSs) are widely distributed among prokaryotes and eukaryotes and have diverse functions in physiology. Recent genome sequencing revealed NOS-like protein in bacteria, but whether these proteins generate nitric oxide is unknown. We therefore cloned, expressed, and purified a NOS-like protein from Bacillus subtilis (bsNOS) and characterized its catalytic parameters in both multiple and single turnover reactions. bsNOS was dimeric, bound L-Arg and generated nitrite from L-Arg when incubated with NADPH and a mammalian NOS reductase domain. Stopped-flow analysis showed that ferrous bsNOS reacted with O2 to form a transient heme Fe(II)O2 species in the presence of either Arg or the reaction intermediate N-hydroxy-L-arginine. In the latter case, disappearance of the Fe(II)O2 species was kinetically and quantitatively coupled to formation of a transient heme Fe(II)NO product, which then dissociated to form ferric bsNOS. This behavior mirrors mammalian NOS enzymes and unambiguously shows that bsNOS can generate NO. NO formation required a bound tetrahydropteridine, and kinetic effects of this cofactor were consistent with it donating an electron to the Fe(II)O2 intermediate and the kinetic effects of this cofactor were consistent with it donating an electron to the Fe(II)O2 intermediate. 

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Nitric-oxide synthases (NOSs, EC 1.14.13.39)¹ are present in insects, mollusks, parasites, fungi, slime molds, and bacteria (1–4). Their amino acid sequences and activities are similar to the mammalian NOSs, suggesting that the mammalian gene came from lower species through evolution. The mammalian NOSs catalyze the oxidation of L-arginine (Arg) to citrulline and NO, with N-hydroxy-L-arginine (NOHA) formed as an enzyme-bound intermediate (5). All mammalian NOSs are bi-domain proteins comprised of an N-terminal oxygenase domain (NOSoxy) that binds protoporphyrin IX (heme), 6R-tetrahydrobiopterin (H4B), and Arg, and a C-terminal flavoprotein domain (NOSred), linked together by a calmodulin (CaM) binding sequence (5). NOS flavoprotein domains are similar to NADPH-cytochrome P450 reductase and related electron transfer flavoproteins (5) and function to provide NADPH-derived electrons to the ferric heme for O2 activation during NO synthesis.

Recent genome sequencing revealed that NOS-like proteins exist in many prokaryotes including Deinococcus radiodurans, Bacillus subtilis, Bacillus halodurans, Bacillus anthracis, ² and Staphylococcus aureus ² (6–8). We recently sequenced, cloned, purified, and characterized D. radiodurans NOS-like protein (deiNOS) whose sequence is 34% identical and 52% conserved to the oxygenase domain of mammalian nitric-oxide synthases (NOSoxy). Purified deiNOS was dimeric, bound substrate Arg and cofactor H4B, and had a normal heme environment, despite its missing N-terminal structures that in NOSoxy bind Zn²⁺, the dihydroxypropyl side chain of H4B, and help form an active dimer in mammalian NOS (4). The deiNOS heme accepted electrons from a separate mammalian NOS reductase and generated nitrite from Arg or NOHA in reactions stimulated by H2B. However, the oxymembrin assay failed to show that deiNOS synthesized NO under these circumstances. Therefore, fundamental questions remain regarding the exact nature of the nitrogen oxide product formed by prokaryotic NOS-like proteins. To help address this issue we characterized the NOS-like protein from B. subtilis (bsNOS). The results establish its Arg and H2B binding, product formation in multiple turnover reactions, nature and kinetics of heme transitions during Arg or NOHA oxidation under single turnover conditions, and unambiguously show that it produces NO as a product.

EXPERIMENTAL PROCEDURES

Materials—All reagents and materials were obtained from Sigma or sources reported previously (9).

Molecular Biology—The NOS gene of B. subtilis (ATCC) was amplified by PCR from genomic DNA. PCR primers generated a NdeI site before the 5′ start codon and a BamHI site after the 3′ stop codon and the amplified fragment cloned into a pET15B expression vector. B. subtilis NOS DNA in pET15B vector transformed into Escherichia coli strain BL21 (DE3) for protein expression.

Expression and Purification of bsNOS and nNOSred—bsNOS had a His6 tag attached to its N terminus to aid purification. Proteins were overexpressed in E. coli strain BL21 (DE3) and purified by using chromatography on Ni²⁺-nitrilotriacetic acid resin for bsNOS and 2,5'-ADP-Sepharose for nNOSred as described earlier (4).

1 Preliminary sequence data were obtained from The Institute for Genomic Research website at www.tigr.org.

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1 The abbreviations used are: NOS, nitric-oxide synthase; CaM, calmodulin; nNOSoxy, nNOS oxygenase domain; nNOSred, nNOS reductase domain; bsNOS, B. subtilis NO synthase; H2B, (6R)-5,6,7,8-tetrahydro-1-biopterin; THF, tetrahydrofolate; NO, nitric oxide; NOHA, N-hydroxy-L-arginine; deiNOS, D. radiodurans NO synthase; iNOSoxy, iNOS oxygenase domain; EPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; DTT, dithiothreitol.
Arg Binding—Arg binding affinity was studied at 25 °C by perturbation difference spectroscopy using 10 mM imidazole according to methods described previously (4).

NO Synthesis, Nitrite Formation, and Citrulline Production—The steady state activities in the reconstituted system containing bsNOS and nNOSred in a 1:1.5 molar ratio were determined at 25 °C using spectroscopic and high performance liquid chromatography fluorometric assays that were described previously in detail (4).

Measurement of Apparent $K_m$ for H$_4$B or Tetrahydrofolate (THF)—Apparent $K_m$ values were determined by double-reciprocal analysis of the NADPH-dependent nitrite formation against various concentrations of H$_4$B or THF in a reconstitution system containing nNOSred and bsNOS.

Stopped-flow Experiments—Oxygen binding spectra were recorded in a stopped-flow instrument equipped with a rapid scanning diode array device (Hi-Tech MG-6000) designed to collect 96 complete spectra within 144 ms. Rapid scanning experiments involved mixing anaerobic solutions containing dithionite-reduced bsNOS, 40 mM EPPS buffer, pH 7.6, 0.5 mM DTT, 150 μM NaCl, and 1 mM Arg or NOHA with airsaturated buffer solutions at 10 °C in the presence or absence of 100 μM H$_4$B or THF. Formation and decay of the Fe(II)-O$_2$ complexes were followed at 410 or 440 nm (10). Signal-to-noise ratios were improved by averaging 10 individual traces. The diode-array data were then fit to different reaction models by a Specfit program from Hi-tech Ltd. to obtain the calculated number of species, their individual spectra, the concentration of each species versus time, and rate constants for each transition.

RESULTS AND DISCUSSION

Primary Structure Analysis—We identified a 1-kb DNA segment that coded for a 369-amino acid bsNOS protein that has 35% identity and 49% conservation with deiNOS and 42% identity and 55% conservation with mouse iNOSoxy. The similarities among the primary sequences of bsNOS, deiNOS, and mouse iNOSoxy are shown in Fig. 1A. Structural elements that make up the iNOS catalytic core are well conserved in bsNOS. This includes residues that contact the heme, bind the pteridine ring of H$_4$B, and position substrate Arg. Like deiNOS, a notable similarity is that bsNOS is missing an extended portion of N-terminal sequence found in the mammalian enzymes. In mammalian NOSs this region codes for an N-terminal hook, a Zn$^{2+}$-binding site, and contains residues that participate in forming the dimer interface and in binding the dihydroxypropyl side chain of H$_4$B (Fig. 1A). Thus, bsNOS is a heme protein similar to mammalian NOSs that also contains residues likely to generate functional Arg and H$_4$B binding sites.

Physical and Spectral Characteristics of bsNOS—Recomb-
nant purified bsNOS migrated in a denaturing SDS-PAGE gel at a molecular mass of ~40 kDa, identical to its cDNA calculated molecular mass. Its migration in a gel filtration column indicated that bsNOS was predominantly dimeric in its native form (data not shown). Thus, bsNOS is a homodimer despite its missing N-terminal elements that stabilize dimeric structures of mammalian NOS proteins (11). Apparently, differences among amino acids in the subunit interface and elsewhere must minimize the relative importance of the N-terminal elements on dimer stability. bsNOS should therefore be valuable for identifying new residues and regions of importance.

Spectral changes obtained upon Arg or H₄B binding are shown in Fig. 1B. Ferric bsNOS showed characteristic absorbance maxima at 460 and 380 nm, indicating its heme bound DTT to form a bisthiolate species identical to mammalian NOS (12). Adding H₄B caused a spectral transition that generated a broad Soret peak at 400 nm, indicating displacement of DTT ligand. Adding Arg caused the Soret peak to shift to 393 nm, indicating that Arg fully displaced DTT and stabilized the bsNOS heme in a five-coordinate high spin state. These spectral properties show that bsNOS binds H₄B and Arg and has a similar heme environment compared with mammalian NOS.

We next monitored Arg displacement of heme-bound imidazole to determine the Arg binding affinity of bsNOS. Upon sequential addition of Arg to H₄B-saturated bsNOS, there was a concentration-dependent spectral shift that indicated Arg could achieve a complete displacement of heme-bound imidazole (data not shown). The apparent k_s value for Arg in presence of 10 mM imidazole and 20 μM H₄B was derived by double-reciprocal analysis and was 50 ± 5 μM in bsNOS, compared with 97 ± 10 μM in deiNOS and nNOSoxy, respectively (Table I). We conclude that Arg binding affinity of bsNOS is similar to mammalian NOS, consistent with its containing a conserved glutamate residue essential for high affinity Arg binding in mammalian NOS.

**Catalytic Activity in Reconstituted Systems—NO synthesis by animal NOSs involves electron transfer between reductase and oxygenase domains in a NOS dimer. Because bsNOS lacks an attached reductase domain, its heme can only receive electrons from a separate donor protein. The Bacillus genome contains several electron transfer proteins, including an FAD- and FMN-containing NADPH oxidoreductase that is similar to the mammalian enzyme cytochrome P450 reductase (13). We converted to stable ferric enzyme that displayed a Soret peak at 427 nm in both cases. The transient species played a Soret peak at 408 nm in both cases. This species converted within 120 ms after mixing into a transient species of mammalian NOS proteins (11). Apparently, differences among amino acids in the subunit interface and elsewhere must minimize the relative importance of the N-terminal elements on dimer stability. bsNOS should therefore be valuable for identifying new residues and regions of importance.

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**Affinity toward H₄B and Tetrahydrofolate—**Using the reconstitution system we determined an apparent Kₘ value of 100 nM for bsNOS versus 30 ± 10 nM for nNOSoxy (Table I). Thus, bsNOS affinity toward H₄B is greater than deiNOS and approaches that of nNOSoxy or other mammalian NOSs whose apparent Kₘ values range between 50 nM to 1 μM. THF is a tetrahydropteridine that can also support catalytic activities of deiNOS (4). The bsNOS was found to productively bind THF with an apparent K_v value of 0.4 μM, which is 50 times lower than that of deiNOS (20 ± 5 μM). Thus, bsNOS also differs from mammalian NOS in being capable of utilizing THF in place of H₄B to support catalysis.

The primary structure of bsNOS is entirely consistent with its behavior toward H₄B and THF. Several residues that surround the H₄B ring in mammalian NOSs and position it near the heme are all conserved in bsNOS. Earlier we thought that deiNOS had poor affinity toward H₄B due to the absence of N-terminal residues that in animal NOSs bind the 6-dihydroxypropyl side chain of H₄B (11). However, bsNOS also lacks this N-terminal region but has affinity similar to mammalian NOS. This suggests that poorer H₄B affinity of deiNOS may be due to other structural differences, for example the unique absence of β4a, β4c, and β5 helix elements in deiNOS. In any case, the missing N-terminal region probably allows bsNOS to bind THF, which contains a bulky substituent in place of the 6-dihydroxypropyl side chain of H₄B.

**Reaction of Ferrous bsNOS with O₂ in the Presence or Absence of H₄B—**During NO synthesis formation of a transient Fe(II)O₂ intermediate is prerequisite for catalysis (10, 14). We utilized rapid-scanning stopped-flow spectroscopy to examine spectral and kinetic properties of the Fe(II)O₂ intermediate in bsNOS and observed whether H₄B or THF would affect its properties. An anaerobic solution of dithionite-reduced bsNOS containing Arg with or without H₄B was rapid-mixed with air-saturated buffer at 10 °C. The initial ferrous species displayed a Soret peak at 408 nm in both cases. This species converted within 120 ms after mixing into a transient species with Soret peak at 427 nm in both cases. The transient species converted to stable ferric enzyme that displayed a Soret peak at 393 nm and visible absorbance band at 650 nm (data not shown). Thus, bsNOS formed a transient Fe(II)O₂ intermediate that is quite similar to that of deiNOS or nNOSoxy (4, 10). The formation and decay kinetics of the Fe(II)O₂ intermediate were determined by monitoring absorbance change at 407 or 440 nm versus time. The direction of absorbance change at these two wavelengths was reversed as expected but otherwise proceeded with identical kinetics (data not shown), as found previously for deiNOS or nNOSoxy (4, 10). Spectral change during Fe(II)O₂ formation or disappearance was described by a single exponential equation in all cases, suggesting both transitions are monophasic. Rates of Fe(II)O₂ formation and decay

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**TABLE I**

| Enzyme       | Turnover number | Kₘ for H₄B | Kₘ for THF | kₜ for Arg |
|--------------|----------------|------------|------------|------------|
| bsNOS        | 11 ± 1         | 0.1 ± 0.02 | 0.4 ± 0.05 | 50 ± 5     |
| deiNOS       | 31 ± 3         | 10 ± 2     | 20 ± 5     | 97 ± 10    |
| nNOSoxy      | 10 ± 1         | 0.03 ± 0.01| >10,000    | 55 ± 4     |
in the presence of Arg with or without H4B are listed in Table II. The rate of Fe(II)O2 formation was somewhat slower in both bsNOS and deiNOS compared with nNOSoxy at the O2 concentration used here, and this rate was unaffected by added H4B in all proteins, consistent with H4B not significantly affecting O2 binding kinetics in animal NOSs (10). The rate of Fe(II)O2 disappearance in H4B-free bsNOS was three times faster compared with nNOSoxy. Adding H4B to bsNOS increased this rate by about 14-fold. Thus, H4B sped Fe(II)O2 disappearance in bsNOS as it does in nNOSoxy (Table II). Because this kinetic effect has been linked to reduction of Fe(II)O2 by H4B in mammalian NOS (12, 14), our results imply that H4B can perform an identical redox function in bsNOS.

Heme Transitions during NOHA Oxidation by Ferrous bsNOS—To examine formation and reactivity of the Fe(II)O2 species in the second step of NO synthesis, we utilized rapid-scanning stopped-flow spectroscopy to identify consecutive heme transitions that occur during oxidation of NOHA in a single turnover reaction. A solution of ferrous bsNOS saturated with H4B and NOHA was rapidly mixed with air-saturated buffer at 10 °C. Global analysis of the rapid-scan data showed that it best fit to a reaction model A to B to C to D. The calculated spectra for species A–D are shown in Fig. 2A. The initial ferrous enzyme (species A) displayed a Soret peak at 408 nm, consistent with the spectrum of ferrous mammalian NOS taken under identical conditions (10, 15). Species B has absorbance maxima at 427 and 555 nm, consistent with the spectrum of the nNOSoxy Fe(II)O2 intermediate at 10 °C in presence of H4B and NOHA (10, 15). Species C has absorbance maxima at 440, 547, and 585 nm, identical to the spectrum of the ferrous mammalian NOS enzyme (12, 16). In mammalian NOSs, H4B is implicated as an electron donor (14, 17) and was recently shown to provide an identical redox function in bsNOS.

In a replica NOHA reaction run without H4B we observed no heme-NO complex or citrulline production, although an identical Fe(II)O2 intermediate was generated (data not shown). Thus, H4B is obligatory for NO synthesis by bsNOS in the single turnover reaction, as is true for mammalian NOSs (12, 14). In mammalian NOSs, H4B is implicated as an electron donor (14, 17) and has been shown to provide an identical redox function in bsNOS.

**Implications for bsNOS Function**—During NOHA oxidation by bsNOS the heme-NO complex forms an identical redox function during its catalysis. Indeed, the B. subtilis genome contains all enzymes needed to synthesize H4B from its GTP precursor (7). Thus, the implication of our current work is that NO synthesis by bsNOS proceeds through the same mechanism as in mammalian NOS and may employ a tetrahydropteridine to provide the second electron required for O2 activation.
Bacillus subtilis NO Synthase Protein

lian NOSynoxy, which has ranged from 2 to 5 s\(^{-1}\) at 10 °C (15, 18). This difference is important, because it sets the maximum rate for NO release from bsNOS during steady state NO synthesis. Moreover, it constrains the rate of ferric heme reduction to remain near or below 0.2 s\(^{-1}\) if the enzyme is to release NO. This is because when the rate of ferric heme reduction exceeds NO dissociation, a majority of the Fe(III)NO product becomes reduced to a ferrous heme-NO species instead of releasing NO (19, 20). Dissociation of NO from the ferrous heme-NO complex is very slow (21), so instead the complex reacts with O\(_2\) to generate higher N-oxides like peroxynitrite or nitrate (19, 20, 22). Thus, our kinetic analysis predicts that bsNOS will either have a relatively slow NO production during steady state due to its slow Fe(III)NO dissociation or will generate higher N-oxides in place of NO if ferric heme reduction as catalyzed by its native redox partner exceeds 0.2 s\(^{-1}\).

The constraint on heme reduction rate caused by slow Fe(III)NO dissociation will also prevent bsNOS from coupling its Fe(II)O\(_2\) formation to substrate oxidation, unless it receives a second electron more quickly than the first during O\(_2\) activation. Consider that the bsNOS Fe(II)O\(_2\) intermediate is inherently unstable as demonstrated by a decay rate of 0.4 s\(^{-1}\) in the Arg-bound, H\(_4\)B-free enzyme (Table I). Thus, for bsNOS to perform substrate oxidation instead of superoxide production, the Fe(II)O\(_2\) intermediate needs to receive an electron at a rate greater than 0.4 s\(^{-1}\). But such high rates would compromise NO release from bsNOS due to its slow NO dissociation as explained above. bsNOS probably solves this paradox by using H\(_4\)B, THF, or a similar donor as a source of the second electron, because they appear to reduce the Fe(II)O\(_2\) species at rates that exceed its oxidative decay (Table I). Thus, by having a tetrahydropteridine provide the second electron during O\(_2\) activation, bsNOS can couple its Fe(II)O\(_2\) formation to substrate oxidation and employ a ferric heme reduction rate that is slow enough to allow for NO release.

In sum, NO synthesis by a prokaryotic NOS-like protein is now established, but a number of related issues still need to be explored. These include determining (i) the crystal structure of a prokaryotic NOS, (ii) the native redox partner(s), (iii) what N-oxide product is generated during steady state synthesis, (iv) what conditions induce expression of these proteins in the bacterium, and (v) if allosteric factors exist that modulate kinetic parameters of the enzyme, as occurs in soluble guanylate cyclase (23). Continued investigation should provide deeper understanding of NOS structure-function and the evolutionary consequence of the NOS gene.

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