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ENZYMOLOGY OF PROTEINS INVOLVED IN NITRIC OXIDE PRODUCTION IN BACTERIA AND PLANTS

A Dissertation
Presented to the Faculty of the Graduate School
of Cornell University
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

by
Jawahar Sudhamsu
May 2009
Mammalian Nitric Oxide Synthases (mNOSs) are heme-based mono-oxygenases, that catalyze the two-step, five-electron oxidation of L-Arginine (Arg) to citrulline and nitric oxide (NO), via the stable intermediate, N⁰-hydroxy-L-Arginine (NOHA). The NOS reaction cycle is well understood, although the identity of the highly reactive, unstable heme-oxy species responsible for substrate oxidation remains relatively unknown. Several gram-positive bacteria contain a homolog of the catalytic domain of mNOSs. With the aim of studying more stable reaction intermediates in substrate oxidation by NOS, we characterized a thermostable NOS homolog from G. stearothermophilus (gsNOS). SDS-PAGE revealed two forms of the enzyme during purification. Resonance Raman spectroscopy of gsNOS-Arg complex revealed bands typical for free-base porphyrin in addition to those typical for heme-incorporated NOS. Co-expression of gsNOS with ferrochelatase resulted in complete heme binding. GsNOS indeed forms highly stable heme-oxy complexes in the presence of substrates. The 3.2 Å X-ray crystal structure of gsNOS reveals a constrained active site that may explain the increase in lifetime of heme-bound ligands to seconds at 4 °C. The slow kinetics of gsNOS offer promise for studying downstream intermediates involved in substrate oxidation by both spectroscopy and crystallography, although the latter was limited by low resolution crystals. Side chain entropy reduction was employed to generate two alternate crystal forms of gsNOS that increase
resolution of the structure for further crystallographic studies. To analyze the heme-oxy intermediates and reaction mechanism, we employed EPR and ENDOR spectroscopy of cryo-reduced oxy-gsNOS-substrate complexes. The data suggested that a heme-ferryl-oxo intermediate oxidizes Arg to NOHA and a heme-peroxo intermediate oxidizes NOHA to NO and citrulline.

AtNOS1, a plant protein was earlier identified as a NOS, but the claim has been questioned. To better understand this enzyme, we characterized the activity and solved the crystal structure of YqeH, a bacterial homolog of AtNOS1 from G. stearothermophilus. Our analysis suggests that YqeH is a GTPase that binds RNA. yqeH complements the morphological defects of atnos1 mutant plants, which indicates that AtNOS1 is also an RNA binding GTPase and not a NOS.
BIOGRAPHICAL SKETCH

Sudhamsu was born in Kaikalur, India in 1980 to his parents, who were both high school teachers. He grew up in several parts of India, because of the transferable nature of his parents' jobs. When he was 5 years old, he followed his parents to Soviet occupied Afghanistan in winter of 1985, where he was introduced to freezing cold winters and the beauty of Kabul. In 1988, due to evacuation of its citizens by the Indian government, following the rise of Mujahideen and withdrawal of Soviet forces, Sudhamsu moved back to his home state in India, and lived in several places across the state until college. In 1998, he moved to Bombay, after being selected for admission into the prestigious undergraduate program at Indian Institute of Technology Bombay (IITB). He joined the 5-yr Integrated Masters program in the Department of Chemistry at IITB, where he found his passion for Biochemistry in courses taught by Dr. K. K. Rao, Dr. C. P. Rao, and Dr. A. K. Lala. During his last two years at IITB, Sudhamsu got interested in Theoretical Chemistry and worked on proteins involved in photosynthesis as a part of his Masters thesis. In early 2003, Sudhamsu was offered admission into the Graduate program at the Department of Chemistry and Chemical Biology at Cornell University. In July '03, he moved to Ithaca, NY and has been working for Prof. Brian Crane on enzymes related to Nitric Oxide production in bacteria and plants since. He plans to continue working in the field of Chemical Biology.
To akka, amma and ammamma for their love and inspiration
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A lot of people have contributed in visible and not so visible ways to this journey that has lasted five and a half years. I would like to start by thanking my mom and dad for their love, support and all the sacrifices that they made to make sure I had all the opportunities to be able to get a higher education than they did. It is because of their dreams and aspirations that I am at the cusp of earning a Ph. D.

I would like to thank my mentor Brian Crane for accepting me into his research group during my first year. I cannot imagine a work environment I could have been in that would have suited me better. I would also like to thank him for constant encouragement and guidance and for giving me several opportunities for professional growth over the past five years. I would like to thank Joanne for teaching me everything I know about molecular biology and for always being very nice to me. I want to thank ex-graduate students from our group, Kartikeya, Sang and Jane for patiently training me in the lab procedures and for inspiring me during my initial months in the Crane group.

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CHAPTER 1

DIVERSE FUNCTIONS FOR PROKARYOTIC NITRIC OXIDE SYNTHASES

1.1 ABSTRACT

Nitric oxide synthases (NOSs) are heme-based mono-oxygenases that oxidize L-arginine to nitric oxide (NO), a signaling molecule and a cytotoxic agent in higher organisms. Although NOS-like activity has been reported in many bacterial lysates, homologs of mammalian NOSs (mNOSs) have been characterized in a much smaller subset of bacteria. These enzymes possess only the catalytic domains of mNOS, but produce NO when suitable reductants are supplied. A notable exception is NOS from a gram-negative bacterium that also appears to contain a reductase module, but one that differs from those found in mNOSs. Crystallographic / spectroscopic analyses of bacterial NOSs bound to substrates and inhibitors have provided insights into properties essential for NO production by this family of enzymes. Bacterial NOSs have functions that differ from mNOSs. In certain Streptomyces strains, NOS is involved in nitration of plant toxins. In Deinococcus, NOS associates with an unusual tryptophanyl-tRNA-synthetase and may also be involved in nitration of tryptophan. In Bacillus, NOS is a key factor in protection against oxidative stress, and thereby, a factor in pathogen defense mechanisms. Studies on bacterial NOSs show great promise for mechanistic studies of NO biosynthesis and for unraveling the wide variety of functions of NO in prokaryotes.
1.2 ANIMAL NOS – A STARTING POINT

Nitric oxide synthases (NOSs) are highly regulated, complex enzymes that catalyze the oxidation of L-arginine (L-arg) to nitric oxide (NO) and citrulline(1). NO in mammals is involved in many biological processes that range from regulation of blood pressure to protection against pathogens(2). Mammalian NOSs are homodimers that contain an N-terminal heme oxygenase domain (NOS$_{ox}$) and a C-terminal flavoprotein reductase domain NOS$_{red}$. NOS$_{ox}$ binds L-arg, heme and the redox-active cofactor 6R-tetrahydrobiopterin (H$_4$B) and contains the catalytic center of the enzyme. NOS$_{red}$ has binding sites for FAD, FMN and NADPH and acts as a source of reducing equivalents for oxygen binding and activation. A calmodulin interaction sequence connects the oxygenase and reductase domains and regulates the reduction of NOS$_{ox}$ by NOS$_{red}$ in a calcium dependent manner(1). The catalytic mechanism of NO formation by mNOS involves two heme based oxygenation reactions, with conversion of L-arg to a stable intermediate N$^o$-hydroxy-L-arginine (NOHA) in the first step, and conversion of NOHA to NO and L-citrulline in the second (Figure 1.1)(3, 4).

![Two-step oxidation of L-arginine to L-citrulline and NO](image)

Figure 1.1: Two-step oxidation of L-arginine to L-citrulline and NO
Considerable mechanistic work has been performed on the NOSs, and although there are parallels between the two steps of catalysis and those reactions carried out by the cytochromes P450, many issues in NOS catalysis remain to be resolved, particularly concerning the heme-oxygen intermediates responsible for transforming L-arg and NOHA(5-7). Key to the catalytic cycle is the reductive activation of oxygen bound to the thiolate-ligated heme. In both steps of the reaction, reduction of the ferric thiolate-ligated heme results in formation of the heme-dioxygen complex, which is best described as a ferric-superoxy species (8-12). Notably, both steps of oxygen activation appear to require H_{4}B as an electron donor to the heme(4, 13). In the first step, the reductase domain reduces the H_{4}B^{••} radical(6, 13-19). In the second step, however, a downstream reaction intermediate(18), possibly the ferrous-NO complex(20) reduces the H_{4}B^{••} radical. Cryo-annealing experiments have demonstrated that reduction of the ferric-superoxy species in mammalian NOSs results in formation of a ferric-peroxo species, that rapidly reacts to form products(21). A ferric-heme-hydroperoxo intermediate was not observed with NOS, as it was for related enzymes such as heme-oxygenase and cytochrome P450(22, 23). Little is known regarding the intermediates that follow the ferric peroxo intermediate, and whether they differ in the two distinct catalytic steps. There is some hope that studies of the bacterial NOS homologs may help resolve these issues.

1.3 PROKARYOTIC NOS – BIOCHEMISTRY AND GENETICS

The first report of NOS-like activity in a bacterium came from studies on a *Nocardia* species(24). A 52 kDa enzyme was purified by following
NADPH oxidation activity in the cell lysate. Stoichiometric formation of product citrulline from radiolabeled L-arg was characterized by chromatographic analysis of derivatized products(25). Mammalian NOS inhibitors N-nitro-L-arginine and N-methyl-L-arginine were shown to inhibit product formation. However, none of the Nocardia species whose genomes have been sequenced to date contain a gene similar to that of the animal NOSs. There are a number of similar reports, where several bacterial strains have been demonstrated to have NOS-like enzyme activity, but the associated genomes contain no obvious NOS homolog. For example, in Lactobacillus fermentum(26) NO production was detected by the conversion of metmyoglobin to nitrosylmyoglobin. Nitrite (an oxidized end product of NO formation) was also shown to build up in the culture media. The amount of nitrite formed was increased two-fold on addition of the mNOS cofactors NADPH, FAD, FMN and H₄B(26), but ^1⁵N nitrite levels only increased by 0.32% when the growth media was supplemented with ^1⁵N-labeled L-arg. To date, no direct formation of NO by a NOS-like enzyme from this bacterium has been shown and in addition, none of the Lactobacillus species sequenced to date contain an mNOS homolog. Similarly, formation of citrulline from both L-arg and NOHA was detected in cell lysates of Rhodococcus sp R312(27). This activity was again inhibited by mNOS inhibitors, and H₄B increased the activity. A Rhodococcus protein (~100 Kda) was recognized by a human iNOS antibody, but not by antibodies raised against bovine eNOS and rat nNOS. L-arg dependent NO production was also shown in Rhodococcus sp. APG1 using the fluorescein-based fluorophore, DAF(28). Whether these bacteria contain NOS is an unanswered question, as their genomes have not yet been sequenced. However, the genome of Rhodococcus sp. RHA1, does contain a
NOS-like sequence. A NOS-like enzyme (~93 Kda) was partially purified from *Salmonella typhimurium* and appeared to produce citrulline and NO(29). But, in this case too, the genome does not contain an enzyme that is an obvious homolog of mNOS. Similar studies have observed NOS-like activities in microbial eukaryotes such as *Entamoeba histolytica*(30) and *Toxoplasma gondii*(31), but again neither of these genomes contain a NOS homolog. A NOS-like enzyme (~64 KDa) was identified from *Staphylococcus aureus* using an mNOS antibody(32), and NOS activity was established. Addition of methylesters of L-arg and N-Nitro-L-arginine to growth media induced protein expression and increased NOS activity(33). This same enzyme, later shown to be a complex of two enzymes: 64 KDa, 67 KDa, was purified from cell lysate and characterized to oxidize L-arg when mNOS cofactors were supplied(34). It was suggested that NOS co-purified with its reductase partner, although no sequence identification of the proteins was reported. It is now well established that *S. aureus* has an mNOS homolog(35-37), but the subunit molecular weight is much less than those reported above. When considering these studies, one should keep in mind that bacteria can and do produce NO from a variety of pathways, many of which are not dependent on NOS. Furthermore, NOS independent chemistry can also convert L-arg to citrulline in the urea cycle, either in one step by arginine deiminase(38) or by arginase / ornithine carbamoyl transferase in two steps(39, 40). In summary, in the absence of complimentary biochemistry and genetics, it can be difficult to convincingly attribute NOS activity to a specific protein.

Nonetheless, there is currently no doubt that bacteria contain NOS-like proteins(41). Genomic sequencing efforts have identified prokaryotic proteins
Figure 1.2: A simplified version of the phylogenetic tree of the various NOSs, with bacteria from each genus shown as one related group. NOS like proteins are found mostly in gram-positive bacteria (blue). A gram-negative bacterium (red) and an archeon (green) also have a NOS-like sequence in their genomes. The groupings are derived from a phylogenetic tree calculated with PHYLIP\cite{87,88}. 32 different NOS sequences from various organisms were aligned using ClustalW. This aligned data was then bootstrapped to generate 1000 different alignments. A phylogeny estimate was then found for each of these data sets using the parsimony method and 1000 phylogenetic trees were constructed. A consensus tree was then generated using the 1000 trees, using the majority rule. Additionally, a comparison of the phylogenetic tree constructed as described in the text was done using 18 bacterial and 1 archael 16S ribosomal RNA sequences (B) to the phylogenetic tree constructed using NOS\textsubscript{ox} sequences the same organisms (C). The difference in the trees in (B) and (C) suggests horizontal transfer of at least some genes.
with high sequence similarity to the oxygenase domains of mNOSs(42, 43). These NOS$_{ox}$ like proteins mostly occur in gram-positive bacteria, although a gram-negative bacterium and an archaeon also have a NOS sequence (Figure 1.2 A). A phylogenetic tree produced from the sequences of 32 NOSs (26 gram positive bacteria, 4 eukaryotes, 1 gram negative bacterium, 1 archeon) (Figure 1.2 A). The bacterial genera Bacillus, Geobacillus, Staphylococcus form a sub-group and are separated from the NOSs from the archaeon N. pharaonis, eukaryotes, the gram negative bacterium S. cellulosum and the bacterial genera Rhodococcus, Streptomyces and Deinococcus (the latter three form another sub-group). NOSs from two species of Exiguobacterium currently have the most divergent sequences when compared to other NOSs. Nonetheless, the NOS$_{ox}$ sequences from these various organisms are reasonably similar (expectation value: ≤ e$^{-95}$), with nearly all heme binding and active site residues conserved among the sequences. We also constructed a phylogenetic tree using 16s rRNA sequences from 17 gram-positive bacteria, 1 gram-negative bacterium and 1 archeon (Figure 1.2 B), and compared it to the phylogenetic tree constructed using NOS sequences from the same organisms (Figure 1.2 C). We did this analysis with a smaller sub-set of sequences as rRNA comparable to bacterial 16s rRNA does not exist in eukaryotes. The grouping of organisms shown in Figure 1.2 C is quite different from the one derived from 16s rRNA sequences in Figure 2B, and thus, the NOS genes are probably similar because they have been recently shuffled in the pangenome and have not had a long time to diverge from each other.

In bacteria, the position of a gene on a chromosome can often give insight into function, provided some of the surrounding genes can be assigned
to a certain pathway or cellular mechanism. The genes surrounding nos are generally the same within each genera, but different among the genera. Unfortunately, there appears to be no obvious significance to the gene arrangements, with exception of Streptomyces. The nos gene in certain Streptomyces strains lies on a pathogenicity island that comprises genes that are involved in the biosynthesis of thaxtomin, a set of phytotoxins that allow the bacteria to infect plants and cause for example potato scab disease. The position of the nos on this transferable pathogenicity island belies its role in thaxtomin biosynthesis (see below).

1.4 BACTERIAL NOS – ENZYMEOLOGY AND STRUCTURE

Spectroscopic properties, structures and catalytic profiles of bacterial NOSs are for the most part very similar to mNOS, with a few interesting exceptions (35-37, 44-53). Sequences of bacterial NOSs are closely related to mammalian NOS$_{ox}$ domain but lack the associated NOS$_{red}$ and an N-terminal hook region that coordinates a zinc ion in mNOS$_{ox}$. Despite these missing regions, bacterial NOSs are dimers, have normal heme spectroscopic properties, bind substrate L-Arg, and produce nitrogen oxide species in an pterin dependent manner (either H$_2$B or the related cofactor tetrahydrofolate, THF). Nonetheless, there are subtle differences among the spectroscopic properties of the heme centers. In particular, B. subtilis NOS (bsNOS) and B. anthacis NOS (baNOS) have a somewhat novel heme environment in their substrate-free forms which may reflect important differences in reactivity and function(51, 52). The structures of B. subtilis NOS complexed with L-Arg and THF(46), S. aureus NOS (saNOS) complexed with S-ethyl-isothio-urea and
NAD⁺(35), and *G. stearothermophilus* NOS (gsNOS) complexed with L-Arg(53) confirmed that the bacterial NOSoxy like proteins are structurally similar to mammalian NOSox with the exception of the missing N-terminal region (Figure 1.3)(46).

![Diagram](image)

**Figure 1.3:** Structures of the NOSox domains of (A) Human inducible NOS with L-arg, heme and H₂B bound; (B) B. subtilis NOS with L-arg, heme and THF bound; (C) S. aureus NOS with S-ethyl-isothio-urea (Seitu, an L-Arg analog), Heme and NAD bound; and (D) G. stearothermophilus NOS with L-arg and heme bound. All structures show high similarity with the exception of the missing N-terminal hook and zinc-binding region in the bacterial NOSs.

Interestingly, the *Streptomyces* NOS may contain an N-terminal zinc site in which one of the two conserved Cys is replaced by a His residue(54). Conservation of all key catalytic residues among bacterial and mammalian
NOSs suggested that all bacterial NOSs produce nitrogen oxides from L-Arg and NOHA(46). Bona fide NO synthesis has been demonstrated for bsNOS(44), drNOS(45) (D. radiodurans NOS) and gsNOS(53). Lack of the N-terminal zinc-binding region allows bacterial NOSs to bind larger pterin cofactors than the mNOSs (i.e. THF)(45, 46). Although most of the prokaryotes that have the nos gene contain some of the H₄B biosynthesis genes as a part of the folate biosynthesis pathway, only the *Bacillus* genus has an obvious homolog of sepiapterin reductase, the enzyme needed for the final step in H₄B biosynthesis: the two step conversion of 6-pyrovoyl-5,6,7,8-tetrahydrobiopterin to 5,6,7,8-tetrahydrobiopterin (H₄B). On the other hand, all organisms produce THF, which provides the same reduced pterin ring structure as H₄B. BsNOS, drNOS and gsNOS have been shown to produce NO only in the presence of either H₄B or THF, with both bsNOS and drNOS showing slightly higher affinity for H₄B(44, 45, 53).

The bacterial NOSs have served as useful subjects for study of mechanism of NO biosynthesis by this general class of enzyme. Nitrosyl-heme complexes of bsNOS bound to both L-Arg and NOHA have been used to mimic the reactive ferrous-heme oxy species that precede substrate oxidation(55). These structures suggest that L-Arg facilitates hydrogen-bonding interactions to the terminal oxygen of the ferrous oxy complex, whereas NOHA, which is protonated on the oxime nitrogen when bound to the enzyme, directs hydrogen bonds to the heme-proximal oxygen. The implication is that on further heme-oxy reduction, NOHA may stabilize a ferric-peroxo intermediate, whereas L-Arg may facilitate break down to a ferryl-oxo species, analogous to peroxidase compound I. Resonance Raman
studies of *Staphylococcus aureus* NOS heme-oxy complexes are consistent with L-arg and NOHA differentially controlling the stability, and perhaps fate, of oxygen intermediates in NOS catalysis(36, 37, 48, 49). Indeed, consistent with the structural studies on the nitrosyl complexes, L-arg weakens the O-O bond of the ferric-superoxo complex in *S. aureus* NOS, whereas, NOHA has little effect on the O-O bond, but rather strengthens the Fe-O bond. Another key difference between bsNOS and mNOS, is that product NO release rates from heme are considerably lower in bsNOS(44). This kinetic difference correlates with a structural difference between in the active center, where a conserved valine residue that resides over the oxygen binding site is substituted by a conserved isoleucine in bacterial NOSs(46). Indeed, switching of this residue in bacterial and mNOSs reverses the trend in heme ligand disassociation rate(56, 57). GsNOS, derived from the thermophile *G. stearothermophilus* has especially stable heme-oxygen intermediates at room temperature, possibly due to a conformational difference as compared to bsNOS, which brings this conserved isoleucine even closer to the oxygen binding site(53).

The bacterial NOSs have also aided in the characterization of partially unfolded structural states common to mNOS$_{\alpha}$ that are involved in enzyme assembly and possibly regulation. mNOS$_{\alpha}$ is known to form a partially unfolded dimer in the absence of H$_3$B and substrate(58, 59). This structural state has a more exposed heme, is more susceptible to proteolysis, and a lower heme redox potential compared to a substrate and pterin bound enzyme(59). BsNOS was crystallized in this form and its structure revealed that the dimer interface and the cofactor binding site is highly disordered(60). These structural changes propagate to nearby helical regions and the active site,
influencing substrate binding. Addition of cofactor and substrate to the crystallization conditions converts this state into a tight, catalytically competent dimer. This “loose dimer” state may be important for limiting heme-based oxygen reduction in the absence of cofactor and substrate.

1.5 REDUCTASE PARTNERS

NO production by NOS from gram-positive bacteria has so far been characterized to be similar to that of mNOS, with the exception that the reducing equivalents cannot be supplied from a covalently attached reductase domain. A *B. subtilis* flavodoxin YkuN was demonstrated to be an efficient electron donor for bsNOS that could support NO synthesis in vitro(61). However, when the *ykuN* gene is deleted, *B. subtilis* still presents phenotypes indicative of NOS activity (see below). Furthermore, bacterial NOSs expressed in *E. coli* produce NO in vivo presumably utilizing heterologous reductases(62). Thus, unlike the mammalian proteins, which contain a dedicated reductase module, the bacterial proteins may be capable of accepting electrons from a number of different reductase proteins(62). Nonetheless, it may still be true that particular functions require specific, yet to be determined, reductases.

A *nos*-like gene identified in the recently sequenced genome of a gram-negative bacterium, *Sorangium (Polyangium) cellulosum*(63) provides a new twist on the redox partners of NOS<sub>ox</sub>. Interestingly, the *S. cellulosum* NOS (scNOS) is the only bacterial NOS so far that has a covalently attached reductase module. However, the domain organization in scNOS is quite
different compared to mNOSs, and the redox active cofactors predicted to be employed by the reductase domain are also unique (Figure 1.4). The scNOS domain structure suggests a variation on the flow of electrons found in mNOS. ScNOS (1163 amino acids) has the first ~450 amino acids with unknown function, followed by a predicted Fe₃S₃ cluster, then by a FAD binding motif, an NAD binding motif and finally a C-terminal NOSox.

![Diagram of domain architecture of NOSs](image)

Figure 1.4: A comparison of the domain architecture of NOSs from different sources. Eukaryotic NOSs have an isozyme specific N-terminal region (which can contain localization motifs), followed by a zinc binding region, a NOSox domain that binds heme, L-arg and H₄B, a calmodulin-binding region and a NOSred that has subdomains that bind FMN, FAD and NAD. NOSs from gram-positive bacteria only possess the NOSox domain (Heme, L-Arg, H₄B or THF), apart from Streptomyces turgidiscabies, which may have a zinc-binding region at its N-terminus. The only gram-negative bacterium known to have a NOS, S. cellulosum, has a novel domain architecture with an N-terminal region of unknown function, followed by a NOSred that contains sub-domains for binding an Fe₃S₃ cluster, FAD and NAD and then a NOSox.

Thus, compared to mNOS, the reductase domain of scNOS is N-terminal, rather than C-terminal to NOSox and the flavodoxin module in mNOS is replaced by an FeS cluster, which will also be capable of one electron chemistry. The FAD-binding ferredoxin reductase module appears to be conserved by scNOS, allowing NAD(P)H to be the ultimate electron donor. In the mNOS field there is considerable interest in how motions of the FMN
domain are linked to reduction of NOS\textsubscript{ox}, largely because this process is the point of regulation by Ca\textsuperscript{2+}-CaM(64-69). Given the novel reductase module and juxtapositions of cofactors in scNOS, understanding NOS\textsubscript{ox} reduction in this interesting variant may shed light on the essential aspects of communication between NOS\textsubscript{ox} and NOS\textsubscript{red}.

1.6 FUNCTIONS OF BACTERIAL NOS

Many years prior to the discovery of the mammalian NOSs bacteria have been known to produce NO. NO was first identified as a biological product in 1967, when it was shown to be an intermediate in anaerobic denitrification in the marine bacterium *Pseudomonas perfectomarinus*(70). NO was found to be released transiently when cell lysate from the denitrifying cultures which were incubated anaerobically with nitrate, malic acid and reducing cofactors NADPH, FAD and FMN(70). It is now well known that during denitrification NO is produced from Cu or Heme-dependent nitrite reductases(71). NO from host and environmental sources is an important signal for many bacteria, and currently a number of NO-specific sensing systems have been identified(72, 73). However, only a small subset of bacteria contains a NOS, and until recently the functions of these enzymes were unknown. NOS derived NO production *in vivo* has been demonstrated for *S. turgidiscabies, B. subtilis, B. anthracis*(74-76). NO in eukaryotic systems encompasses a wide array of functions in processes that are mostly involved in inter-cellular communication or protection against pathogens. Taken as a whole, NO appears to have a variety of functions in prokaryotic systems as well. Indeed, in what we know of bacterial NOS function, different processes
rely on NOS-derived NO in bsNOS, drNOS and stNOS (NOS from *S. turgidiscabies*).

**1.6.1 Streptomyces turgidiscabies NOS**

The first indication into bacterial NOS function came from the finding that a NOS open reading frame was contained on a pathogenicity island (Figure 1.5 A) common to certain *Streptomyces* strains that cause potato scab disease[54]. The pathogenicity is derived from a class of phytotoxins called thaxtomin that interfere with plant cell wall synthesis. Thaxtomin are unusual dipeptides (derivatives of cyclo-[L-tryptophanyl-L-phenylalanine]; Figure 1.5 B) produced by non-ribosomal peptide synthesis[77].

![Diagram of thaxtomin producing pathogenicity island](image)

**Figure 1.5:** (A) The thaxtomin producing pathogenicity island in *Streptomyces turgidiscabies*. The genes for nos (blue) along with *p450* (gray), *txtA*, *txtB* and *txtC* (green) cluster on the island. The pink genes represent mobile genetic elements. (B) Structure of Thaxtomin A. NOS is involved in the nitration of the tryptophan moiety (dark blue) at the 4-position.
Most interestingly, the tryptophanyl moiety is nitrated at the 4-position (77). The location of *nos* on the pathogenicity island that mobilizes among species to enable in thaxtomin biosynthesis and its position on the island in close proximity to the two non-ribosomal peptide synthases of the thaxtomin biosynthetic pathway strongly suggested that *nos* might be involved in nitration of thaxtomin. Disruption of the nos gene greatly curtailed thaxtomin production, which was partially restored by nos complementation (54). No non-nitrated derivatives of thaxtomin were detected in the growth medium of the knock-out strain. Inhibitors of mammalian NOSs added to the growth media of *S. turgidiscabies* resulted in a decrease in thaxtomin production without having any effect on the cell growth (54, 78). This attributed the decrease in toxin production to loss of NOS activity. Also, stNOS over-expressed in *E. coli* produced nitrite from NOHA, as detected by a standard assay that measures nitrite production as the final product of NO reacting with oxygen in solution. Finally, it was demonstrated in a feeding study with $^{15}$N-L-arginine (labeled on the terminal guanidinium nitrogens) that the thaxtomin nitro group nitrogen originates from the terminal guanidinium nitrogen of L-arginine. NOS is the only known enzyme that oxidizes a terminal guanidinium nitrogen of L-arginine to NO. Thus, it was concluded that the position of *nos* on the thaxtomin pathogenicity island was because it is a key player in the nitration of the Trp moiety (54). Biosynthetic nitration reactions are rare, and usually involve oxidation of an amine (79). The chemical mechanism of NOS mediated nitration is likely to be complex and may involve other factors because NO is unlikely to react with the indole moiety in tryptophan, although, activated forms of NO, such as nitrosonium
(NO$^-$), peroxynitrite (ONOO$^-$), nitronium (NO$_2^+$), or nitrogen dioxide (NO$_2$) are known to actively nitrate aromatic groups(80, 81).

Interestingly, the *Streptomyces* strains produce NO far in excess of that needed for biosynthesis of the toxin and NO production occurs at the site of infection, such as nascent root tips(74). In plants, NO is known to act as a signal for the growth and extension of such structures(82) – raising the interesting possibility that the pathogen produced NO also promotes the growth of tissue conducive to colonization.

Further biochemical / structural characterization of stNOS and other thaxtomin related enzymes have lagged due to difficulties associated with production of the enzymes in sufficient amounts.

1.6.2 *Deinococcus radiodurans* NOS

With the knowledge that stNOS was involved in biosynthetic nitration reactions, nitration activity was investigated for other bacterial NOSs. Only drNOS was found to catalyze the synthesis of small amounts of 4-nitro-L-Tryptophan *in vitro*, when reductants are supplied by a surrogate NOS$_{rad}$(83). In addition, drNOS co-purifies with an unusual tryptophanyl tRNA synthetase (TrpRS)(84), providing a link to Trp metabolism in *Deinococcus* and *Streptomyces*. This link between drNOS and stNOS correlates well with the close phylogenetic separation between their sequences. *D. radiodurans* has two TrpRS genes, and one of the two, (TrpRS I) is more closely related by sequence to standard bacterial TrpRSs than the other (TrpRS II). Both TrpRS I
and TrpRS II amino acylate tRNA\textsuperscript{Trp}, but TrpRS I is \(~3\times\) more active than TrpRS II\cite{84}. The complex of TrpRS II and drNOS has a higher Trp nitration activity as compared to drNOS alone\cite{83}. The unusual Trp binding site of TrpRS II suggested that Trp analogs with substitutions on the indole moiety could be recognized and potentially coupled to tRNA by this enzyme\cite{85, 86}. Indeed, TrpRS II charges tRNA\textsuperscript{Trp} with either Trp, 4-nitro-Trp or 5-hydroxy-Trp with nearly equal specificity \textit{in vitro}, whereas TrpRS I only charges Trp\cite{85}. Thus, the drNOS:TrpRS II complex can synthesize 4-nitro-Trp-tRNA\textsuperscript{Trp}. The purpose of this unusual product is unclear and currently no evidence exists for the incorporation of 4-nitro-Trp into proteins. Instead, it is more likely that 4-nitro-Trp-tRNA\textsuperscript{Trp} is used by \textit{D. radiodurans} for production of a yet to be discovered secondary metabolite.

\textbf{1.6.3 NOS from \textit{Bacillus subtilis} and \textit{Bacillus anthracis}}

In contrast to the functions of stNOS and drNOS, NOSs from \textit{B. subtilis} (bsNOS) and \textit{B. anthracis} (baNOS) protect the bacteria against oxidative stress\cite{75, 76}. Exposure of cells to mM concentrations of H\textsubscript{2}O\textsubscript{2} leads to hydroxyl radical formation via Fenton chemistry (Figure 6), DNA damage and cell death. However, for \textit{B. subtilis} cells that are pre-treated with \(~30\,\mu\text{M}\) NO 5 sec prior to 10 mM H\textsubscript{2}O\textsubscript{2}, the survival rate increases \(~100\,\text{fold}\)\cite{75}. Addition of NO either simultaneously with H\textsubscript{2}O\textsubscript{2} or after H\textsubscript{2}O\textsubscript{2} does not show this effect\cite{75}. NO is known to activate certain genes in \textit{B. subtilis} and \textit{E. coli} to protect the cells from oxidative and nitrosative stress\cite{75}. However, in this case, since the protection was achieved within 5 sec of NO exposure induction of gene expression could not be the explanation. Rather, a readily available
defense mechanism against oxidative stress must be at play. Gene disruption of nos in B. subtilis produced a strain much more susceptible to oxidative damage under conditions where reduced thiols were up-regulated(75). This lead to the hypothesis that NOS-derived-NO reduces oxidative damage by inhibiting the enzyme that reduce thiols that would normally catalyze Fenton chemistry, thereby produce hydroxyl radicals, resulting in DNA damage (Figure 1.6). In addition, NO directly activated a specific B. subtilis catalase. The pathogenic S. aureus was also shown to exhibit the same NO-mediated thiol-protection system as B. subtilis in similar experiments(75).

![Diagram](image)

**Figure 1.6:** In B. subtilis, exposure to oxidative stress (hydrogen peroxide) results in an Fe$^{2+}$ mediated formation of hydroxyl radicals (Fenton reaction), which cause DNA damage. NO inhibits the formation of reduced thiols that recycle Fe$^{3+}$ to Fe$^{2+}$ and thus protects the organism. NO also activates catalase (not shown) that breaks down hydrogen peroxide to oxygen and water.

Phagocytes produce large amounts of nitric oxide and reactive oxygen and nitrogen species in response to pathogenic infection. Studies similar to the ones above also revealed a similar cyto-protection function for B. anthracis NOS (baNOS)(76). Furthermore, in vivo survival of pathogenic B. anthrasis critically depends on its own NO-synthase activity. Spores of B. anthracis nos knock-out mutant lose their virulence in a mouse model of systemic infection.
and exhibit extremely compromised survival when germinating in macrophages.(76). BaNOS dependent resistance of B. anthracis to oxidative stress from macrophages is thought to originate from NO-mediated activation of a bacterial catalase, like in B. subtilis and suppression of damage by the Fenton reaction. Furthermore, NO production is induced in the pathogen in response to the oxidative burst of the host defense mechanism(76). It is a surprising and intriguing finding that NO protects the bacteria from the host, in the early stages of infection and yet is also produced by the host, as a component of its own protective oxidative burst. More work is needed on the precise role played by NO on both sides of this host pathogen interaction.

1.7 CONCLUSIONS AND LOOKING AHEAD

Bacterial NOSs continue to serve as tractable subjects for unveiling the mechanistic details of NO synthesis. Moreover, cellular studies of bacterial NOSs promise to reveal unanticipated biochemical pathways and new functions for NO in microbial settings. In fact, prokaryotes may employ NOSs as general sources of NO for use in multiple cellular processes, perhaps within the same organism. In this sense, the global functions of bacterial NOSs parallel their mammalian counterparts; however, it is within the specifics of these biological activities where we can hope to discover novel aspects of NO chemistry and biology.
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CHAPTER 2

STRUCTURE AND REACTIVITY OF A THERMOSTABLE PROKARYOTIC NITRIC OXIDE SYNTHASE THAT FORMS A LONG-LIVED OXY-HEME COMPLEX

2.1 ABSTRACT

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 N-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 H₂O₂. 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.

2.2 INTRODUCTION

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 N-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, formation with cofactors other than H₄B, 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 (Figure 2.1), 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 N⁶-hydroxy-L-arginine (NOHA), an enzyme bound intermediate (19), is further oxidized to NO and citrulline.

![Figure 2.1: 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).]
In both the arginine and NOHA reactions, reduction of the Fe(III) heme enables oxygen binding and formation of a heme-dioxygen complex, that is best described as a ferric superoxy species [Fe(III)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$_2$B acts as an electron donor to the ferric-superoxy species in both steps of NO synthesis (Figure 2.1). In the first step the reductase domain reduces the H$_2$B radical (18, 27-33). In the second step, a downstream reaction intermediate (25), possibly a ferrous-heme NO complex (34), reduces the H$_4$B radical. Reduction of the Fe(III)O$_2$ species at cryogenic temperatures results in a ferric heme peroxo 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 iNOSoxy decreases 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 mono-oxygenases, 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) (FeIV=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-oxy 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.
2.3 EXPERIMENTAL PROCEDURES

2.3.1 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).

2.3.2 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.

2.3.3 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 over-expressed 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.
2.3.4 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 the 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.

2.3.5 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 F_{obs} - F_{calc} and 2F_{obs} - F_{calc} maps. Addition of L-arginine, heme and water molecules amidst cycles of refinement produced the final model.

2.3.6 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.

2.3.7 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.

2.4 RESULTS
2.4.1 General properties. GsNOS was PCR cloned and expressed with a His$_6$ affinity tag in E. coli. The protein has 65% sequence identity when compared to the NOS protein from the related mesophile B. subtilis (Figure 2.2). 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).
Figure 2.2: Comparison of bacterial and mammalian NOSX sequences. Sequences for B. Subtilis bsNOS, D. Radiodurans drNOS, G. Stearothermophilus gsNOS, murine inNOS, bovine eNOS and human nNOS are color-coded to highlight Cys ligands for binding zinc and heme iron (yellow letters), L-arginine-binding residues (blue letters), and H4B binding residues (red letters). This figure was adapted from reference (15).
Figure 2.3: 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.

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) (Figure 2.3) 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 (Figure 2.3).
2.4.2 Thermal stability. GsNOS shows high thermal stability. Molar ellipticity measurements ($\theta_{222}$) of NOS dimers indicate irreversible loss of secondary structure with increasing temperature (Figure 2.4).

Figure 2.4: Temperature melting curves for gSNOS (---), gSNOS+13 (- - -) and bSNOS (-----).

We define the melting temperature as that at which half of the ellipticity ($\theta_{222}$) 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 (Figure 2.4). In all cases, loss of secondary structure, as evidenced by CD, is irreversible.
2.4.3 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 2.1).

Table 2.1: Rates of nitrite production by NOSs from various sources as a function of temperature.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>[NO₂⁻] production gsNOS (min⁻¹ heme⁻¹)</th>
<th>[NO₂⁻] production bsNOS (min⁻¹ heme⁻¹)</th>
<th>[NO₂⁻] production deiNOSa (min⁻¹ heme⁻¹)</th>
<th>[NO₂⁻] + [NO₃⁻] production nNOSb (min⁻¹ heme⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 °C</td>
<td>2.6 ± 0.1</td>
<td>4.6 ± 0.1</td>
<td>7.5 ± 0.5</td>
<td>28 ± 3</td>
</tr>
<tr>
<td>35 °C</td>
<td>3.6 ± 0.2</td>
<td>13.3 ± 0.3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>50 °C</td>
<td>10.3 ± 0.1</td>
<td>50.3 ± 1.1</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

a Reference (16)
b Reference (27)

The amount of nitrite formed was quantified 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 NOₓ 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.

2.4.4 Single Turnover Experiments

2.4.4.1 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 (Figure 4) and the line shape was considerably different than substrate free reduced gSNOS, which has a Soret peak at 411 nm and a broad shoulder from 440 nm to 490 nm (Figure 2.5).

![Figure 2.5: 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).](image)

Furthermore, the extinction coefficient for the Soret band of the intermediate is
less than that of both the ferric and ferrous free enzyme. The 413 species was stable for ~1 min at 4 °C, and slowly decayed back to the ferric form (403 nm) with a change in line shape. Conversion to the ferric form of the enzyme was not complete even after 3 min at 4 °C, and required increasing the temperature of the cell to 25 °C. As the new 413 species results from mixing air-saturated buffer with the reduced protein, it likely represents the [Fe(II)O₂] complex; although given the limited time resolution, the initial spectrum observed on addition of oxygen could include some 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 M H₂B, 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 M H₂B, the intermediate decayed to the Fe(III) form within 5 s and it could not be observed by these methods. In presence of H₂B, an oxidized form of H₄B 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.

2.4.4.2 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 (Figure 2.3). 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 (Figure 2.6). 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).

![UV-Vis spectra of the gssNOS heme-oxygen complex](image)

Figure 2.6: UV-Vis spectra of the gssNOS heme-oxygen complex (Absorption maximum at 427 nm) converting to the ferric form (Absorption maximum at 399 nm). Spectra were taken every 5 sec.

H₄B accelerates the decay of the ferrous-oxy species and appearance of the ferric heme species (Figure 2.7). In the presence of 15 μM H₄B, 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 H₄B (Table 2.2).
Figure 2.7: 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.

Increasing the concentration of H₄B 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 2.2). Because of these considerations, and a binding constant of H₄B to B. subtilis NOS (100 nM) (12) that is much lower than the concentrations being used here, the slow phase in the presence of H₄B is unlikely to derive from gsNOS free of H₄B.

With H₄B, 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 2.2). This may indicate that the kinetics of
the optical changes at these two wavelengths are not same when H₂B is present. Such behavior could result from the presence of another intermediate between the ferrous-oxy and Fe(III) states of gsNOS with H₂B. Better time resolution is necessary to address whether this is truly the case.

Table 2.2: 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.

<table>
<thead>
<tr>
<th>System</th>
<th>Fe(III) formation rate (s⁻¹)</th>
<th>Fe(II)-Oxygen decay (s⁻¹)</th>
<th>Fe(II)-Oxygen decay² (s⁻¹)</th>
<th>Fe(II)-Oxygen decay³ (s⁻¹)</th>
<th>Fe(II)-Oxygen decay (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(k x 1000)</td>
<td>(k x 1000)</td>
<td>(k x 1000)</td>
<td>(k x 1000)</td>
<td>(k x 1000)</td>
</tr>
<tr>
<td>gsNOS (4 °C)</td>
<td>40 ± 8</td>
<td>47 ± 5</td>
<td>1370 ± 40</td>
<td>400 ± 200</td>
<td>140</td>
</tr>
<tr>
<td>NOS + Arg</td>
<td>24 ± 3</td>
<td>28 ± 3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NOS + Arg + H₂B</td>
<td>k₁ ≥ 400</td>
<td>k₁ = 1400 ± 150</td>
<td>3900 ± 200</td>
<td>5400 ± 200</td>
<td>10000</td>
</tr>
<tr>
<td>NOS + Arg + H₂B</td>
<td>k₂ = 8 ± 2</td>
<td>k₂ = 76 ± 21</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(f=N.D.)</td>
<td>(f=N.D.)</td>
<td>(f=0.85)</td>
<td>(f=0.15)</td>
<td></td>
</tr>
</tbody>
</table>

² Reference (11)
³ Reference (12)
⁴ Reference (20)
⁵ N.D. not determined due to inaccuracy caused by the rapid decay of the fast component.

In contrast, 15 µM H₂B had a more minor, but opposite effect on the rates for Fe(III) formation or Fe(II)-O₂ complex decay (Table 2.2). 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 H₂B.

2.4.4.3 N-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 H₂B, 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 (Figure 2.8 A). 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 (Figure 2.8 B). Notably, this spectrum only occurs in the

45
presence of both NOHA and $H_4B$.

Figure 2.8: GsNOS converts NOHA to a ferric-NO species in the presence of $H_4B$. UV-Vis spectra taken in 5 s second intervals after addition of airsaturated buffer in the presence of 1 mM NOHA and 60 μM $H_4B$. (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).
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 2.3), 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 2.3).

Table 2.3. Kinetic parameters for single turnover experiments with NOS, NOHA and H₄B

<table>
<thead>
<tr>
<th>Transformation</th>
<th>gsNOS (4 °C)</th>
<th>bsNOS (10 °C)a</th>
<th>iNOS (10 °C)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe(III) formation rate</td>
<td>40 – 80</td>
<td>210 ± 5</td>
<td>2300 ± 100</td>
</tr>
<tr>
<td>(k × 1000) (s⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe(III)-NO decay</td>
<td>50 – 100</td>
<td>210 ± 5</td>
<td>2300 ± 100</td>
</tr>
<tr>
<td>(k × 1000) (s⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Reference (12)  
b Reference (25)

2.4.5 Crystal Structure

At 3.2 Å resolution (Table 2.4), the overall structure of gsNOS (Figure 2.9 A) 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.
Table 2.4. Data collection and Refinement Statistics for gsNOS complexed with L-arginine.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>no. of residues</td>
<td>717</td>
</tr>
<tr>
<td>ligand</td>
<td>L-arginine</td>
</tr>
<tr>
<td>number of waters</td>
<td>223</td>
</tr>
<tr>
<td>resolution (Å)</td>
<td>3.2 (3.20 – 3.29)</td>
</tr>
<tr>
<td>number of unique reflections</td>
<td>15805</td>
</tr>
<tr>
<td>number of observations</td>
<td>60395</td>
</tr>
<tr>
<td>% completeness</td>
<td>92.0</td>
</tr>
<tr>
<td>( \langle</td>
<td>I</td>
</tr>
<tr>
<td>( R_{sym} )^b (%)</td>
<td>17.0 (48.3)</td>
</tr>
<tr>
<td>( R^c ) (%)</td>
<td>22.2 (31.3)</td>
</tr>
<tr>
<td>( R_{free} )^d (%)</td>
<td>29.5 (36.2)</td>
</tr>
<tr>
<td>overall ( \langle B \rangle ) (Å^2)</td>
<td>21.2</td>
</tr>
<tr>
<td>mainchain ( \langle B \rangle ) (Å^2)</td>
<td>18.4</td>
</tr>
<tr>
<td>sidechain ( \langle B \rangle ) (Å^2)</td>
<td>24.4</td>
</tr>
<tr>
<td>rmsd for bonds(^f) (Å)</td>
<td>0.0083</td>
</tr>
<tr>
<td>rmsd for angles(^f) (degrees)</td>
<td>1.511</td>
</tr>
</tbody>
</table>

\(^a\) Intensity of the signal to noise ratio. \(^b\) \( R_{sym} = \Sigma |I_{i} - \langle I \rangle| / \Sigma |I_{i}| \). \(^c\) \( R = \Sigma |F_{obs} - |F_{calc}| / \Sigma |F_{obs}| \) for all reflections (no \( \sigma \) cutoff). \(^d\) \( R_{free} \) calculated against 10\% of reflections removed at random. \(^e\) Overall model average thermal (B) factor. \(^f\) Root mean square deviations from bond and angle restraints. \(^g\) Highest resolution bin for compiling statistics.

GsNOS maintains the overall fold of mammalian NOSoxy enzymes, with a conserved \( \beta \)-winged core surrounded by \( \alpha \)-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 ~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 (Figure 2.9 B).
Figure 2.9: 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 \( C_{\alpha} \) traces of gsNOS (blue) and bsNOS (dark orange). The topologies of the proteins are highly similar, although gsNOS appears slightly more compact.
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 (Figure 2.10).

![Figure 2.10: 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). 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 (Figure 2.10). 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.

2.5 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 (Figure 2.2), 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 *bona fide* NO in single turnover experiments when only NOHA and H$_4$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 *D. radiodurans, B. subtilis* and *S. aureus*. For all thermophiles for which genome sequences are available, only one other, *Geobacillus kaustophilus* also has a NOS gene. GkNOS shares 94% sequence identity with gsNOS, but has a much longer N-terminal extension (~50 residues). *G. kaustophilus* has genes coding for enzymes that synthesize H$_4$B, and the *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 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 peroxy 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 O₂ with CO is thought to be the ferrous-oxygen complex [Fe(II)O₂] (420 nm, 560 nm), whereas, the complex which readily forms in the presence of substrate and does not exchange O₂ with CO is thought to be the ferric-superoxy complex (432 nm, 564 nm). Although, the Fe(II)O₂ and Fe(III)O₂ 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 ferric or ferrous forms of the enzyme. 40 μM H₄B accelerates the rate of decay of this species to the ferric form, whereas H₂B, 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 H₄B accelerates the decay of this species to the ferric enzyme. This is a hallmark for H₄B acting to reduce the Fe(III)-O₂⁻ 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 H₄B. Rates for gain of the Fe(III) (399 nm) and loss of Fe(III)-O₂⁻ (427 nm) may not be the same when H₄B is bound (Table 2). This may indicate additional observable spectral intermediates may manifest between the Fe(III)-O₂⁻ and Fe(III) states when both L-arginine and H₄B 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 2.5). 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 O\textsubscript{2} (35, 57, 58, 72).

Table 2.5. 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.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Temperature (^\circ\text{C})</th>
<th>Soret peak (nm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>nNOS\textsubscript{oxy} + Arg</td>
<td>25</td>
<td>430</td>
<td>(74)</td>
</tr>
<tr>
<td>nNOS\textsubscript{oxy} + Arg</td>
<td>10</td>
<td>427</td>
<td>(20)</td>
</tr>
<tr>
<td>nNOS\textsubscript{oxy} + N\textsuperscript{c}-methyl Arg</td>
<td>-30</td>
<td>419</td>
<td>(22)</td>
</tr>
<tr>
<td>nNOS + Arg</td>
<td>10</td>
<td>427</td>
<td>(73)</td>
</tr>
<tr>
<td>nNOS</td>
<td>-30</td>
<td>416</td>
<td>(21)</td>
</tr>
<tr>
<td>iNOS\textsubscript{oxy} + Arg</td>
<td>10</td>
<td>427</td>
<td>(30)</td>
</tr>
<tr>
<td>eNOS</td>
<td>7</td>
<td>420</td>
<td>(26)</td>
</tr>
<tr>
<td>eNOS + Arg</td>
<td>7</td>
<td>432</td>
<td>(26)</td>
</tr>
<tr>
<td>eNOS + NOHA</td>
<td>7</td>
<td>428</td>
<td>(26)</td>
</tr>
<tr>
<td>P450 (liver microsomal)</td>
<td>25</td>
<td>418</td>
<td>(77, 78)</td>
</tr>
<tr>
<td>P450\textsubscript{soc}</td>
<td>-30</td>
<td>422</td>
<td>(79)</td>
</tr>
<tr>
<td>P450\textsubscript{soc}</td>
<td>-17</td>
<td>420</td>
<td>(80)</td>
</tr>
<tr>
<td>P450\textsubscript{soc} + Cholesterol</td>
<td>-17</td>
<td>423</td>
<td>(80)</td>
</tr>
<tr>
<td>CytP450cam (P. putida)</td>
<td>4</td>
<td>418</td>
<td>(81, 82)</td>
</tr>
<tr>
<td>CytP450 (bacterial)</td>
<td></td>
<td>418</td>
<td>(83)</td>
</tr>
<tr>
<td>Chloroperoxidase</td>
<td>-30</td>
<td>430</td>
<td>(84)</td>
</tr>
<tr>
<td>Chloroperoxidase</td>
<td>25</td>
<td>428</td>
<td>(55)</td>
</tr>
<tr>
<td>gsNOS</td>
<td>4</td>
<td>413</td>
<td>This work</td>
</tr>
<tr>
<td>gsNOS + L-arginine</td>
<td>4</td>
<td>427</td>
<td>This work</td>
</tr>
<tr>
<td>gsNOS + NOHA</td>
<td>4</td>
<td>423</td>
<td>This work</td>
</tr>
</tbody>
</table>

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-oxy 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-oxy 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 N\textsuperscript{\textcircled{C}}-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 O\textsubscript{2} oxygen induced by substrate will favor Fe(III)-O\textsubscript{2} over Fe(II)-O\textsubscript{2} in NOS.

In presence of NOHA and absence of H\textsubscript{4}B, no striking spectral changes are observed after addition of oxygen and conversion to the ferric form of the enzyme follows. Addition of H\textsubscript{2}B 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 H\textsubscript{4}B, 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 H\textsubscript{4}B. As no Fe(III)NO complex forms with H\textsubscript{2}B, this data also corroborates H\textsubscript{4}B 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 2.3).

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 2.2). 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-oxy 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 (Figure 2.2). 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) (Figure 2.10). 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 2.3). 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 (Figure 2.9 B). Surprisingly, removal of N-terminal extension (~13 residues) decreases the T_m 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.

2.6 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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CHAPTER 3

IMPROVING HEME INCORPORATION INTO OVER-EXPRESSIONED PROTEINS WITH CO-EXPRESSION OF FEROCHELATASE

3.1 INTRODUCTION

Heme proteins serve in a wide range of functions such as transport and storage of oxygen, decomposition of reactive oxygen species, catalytic oxidation of substrates, electron transfer, production and sensing of nitric oxide, signal transduction and control of gene expression. Incomplete heme incorporation into recombinant over-expressed proteins is a common problem [1-6], and several strategies have been developed to alleviate this limitation in protein production [4-10]. For example, slowing the protein expression rate so as not to exceed heme biosynthesis increases heme content in the over-expressed heme protein, but reduces protein yield [11]. Addition of δ-amino levulinic acid, a precursor in the C5 heme biosynthesis pathway, to the growth media slightly before or at the time of induction of protein expression increases rates of heme production and levels of incorporation [4,12,13]. For some cases this procedure is sufficient for full heme loading, for others, it is not [7,8,14,15]. Another strategy is to supply the host bacteria, usually E. coli, with excess hemin. However, most E. coli strains do not possess a heme transport system, and thus, uptake of hemin from the growth media relies on diffusion through the cell membrane, which can be severely limiting. As a result, better hemin uptake can be achieved by co-expression of heme transport genes from other gram-negative bacteria with the heme-protein of
interest [6,9,10]. One such example is the heme transport system from *P. shigelloides*, which consists of proteins Hug A/B/C/D, TonB, Exb B/D. Graves et. al.[6] have shown that co-expression of this uptake system along with the target hemoglobin gene, while supplementing the growth media with hemin, results in relatively high amounts of holo-protein compared to the control experiment in which the transport genes were not induced. An estimate of the holo-protein:apo-protein ratio was not reported. Another similar method [9] involves the co-expression of the heme receptor ChuA from *E. coli* strain O157:H7 along with the protein of interest while supplementing the growth media with Hemin. This method also shows a substantial increase in the amount of heme-incorporation, although the ratio of holoprotein:apoprotein was also not reported. Another strategy takes advantage of *E. coli* strain RP523, which is especially permeable to heme and also has the *hem B*, porphobilinogen synthase, gene disrupted. Near stoichiometric incorporation (0.8-1.0 heme per protein) of heme or heme analogs were achieved by simply supplementing the media with the molecule of interest [16].

Obtaining a homogeneous protein sample is essential for characterization by techniques such as X-ray crystallography as often only pure species crystallize. Furthermore, catalytic parameters may also be sensitive to contaminating apoprotein depending on how protein concentration is measured and whether the apoprotein has an inhibitory effect on the sample. The requirement of fully incorporated samples may not be so great for characterization with techniques such as EPR, Mossbauer, as the apoprotein is spectroscopically silent. However, as will be shown below, in addition to less than stoichiometric heme incorporation, recombinant heme proteins can also be loaded with
unmetallated, free porphyrin and such species can interfere in spectroscopic studies. Notably, expression of fully loaded heme proteins is also of commercial interest. The feasibility of recombinant human hemoglobin as an oxygen delivery pharmaceutical is limited by the yield of holoprotein in E. coli [6]. In such cases methods that involve co-expression of several heme transport genes along with the protein of interest (hemoglobin in this case) are expensive with respect to the energy resources available to the cell over-expressing protein. Herein, we put forth an easy and inexpensive method for absolute incorporation of heme proteins that are over-expressed in E. coli. Co-expression of just one protein native to the host cell, ferrochelatase (FC), in the presence of 60 μM (10 mg/L, ~$0.50 per liter of cell culture) δ-ALA is sufficient for 100% heme incorporation. We demonstrate the efficacy of this method for three unrelated heme proteins derived from different organisms.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Materials Sodium Chloride was obtained from Mallinkrodt, Ferric Chloride, IPTG and TRIS were from Fisher Scientific, Kanamycin and Chloramphenicol from USBiological. δ-ALA was obtained from Sigma-Aldrich.

3.2.2 Cloning and expression Genomic DNA was extracted from E. Coli BL21(DE3) cells using the genomic DNA extraction kit from Epicenter. The FC gene was then PCR amplified (Phusion polymerase, New England Biolabs) from E. coli genomic DNA with primers that generated 5’ and 3’ NdeI and XhoI restriction sites, respectively. This FC gene was then cloned into NdeI and XhoI sites in Multiple Cloning Site-2 of pACYCduet vector (Novagen). A
stop codon was introduced in the primer right before the \textit{XhoI} site to express the protein without the S-tag. GsNOS was cloned earlier into pET28-a vector (Novagen) between \textit{NdeI} and \textit{XhoI} sites \cite{18}. The pET28a-gsNOS plasmid was digested using \textit{NcoI} and \textit{XhoI} to include the His-tag and the thrombin cleavage site, and the pACYCduet-Ferrochelatase plasmid was digested using \textit{NcoI} and \textit{SalI}, since \textit{SalI} and \textit{XhoI} both result in compatible cohesive ends. The His-tag, thrombin cleavage site and gsNOS gene containing fragment were then cloned between \textit{NcoI} and \textit{SalI} sites in Multiple cloning site-1 of the pACYCduet-Ferrochelatase plasmid. This resulted in a pACYCduet plasmid that could be used to over-express gsNOS with a cleavable His-tag and ferrochelatase with no tag. GsNOS was expressed and purified as reported before (ref). Co-expression of gsNOS and FC was also performed similar to expression of gsNOS alone, although, a lesser amount of \textit{\delta-ALA} was added at the time of induction (10mg/L versus 25 mg/L for gsNOS), and the growth media was supplemented with 100 \textit{\mu M} FeCl$_3$. The antibiotics chloramphenicol (34 \textit{\mu g/L}) and kanamycin (50 \textit{\mu g/L}) were added to the growth media of pACYCduet-gsNOS-FC and pET28a-gsNOS plasmids respectively. UV-Vis absorption spectra were recorded as reported earlier \cite{18}. Resonance Raman spectra were also recorded as described previously \cite{17}.

3.2.3 \textit{Resonance Raman experiments}. The optical absorption spectra were recorded with a Shimadzu UV2100U spectrophotometer. Resonance Raman spectra were obtained by using 441.6 nm excitation from a He–Cd laser (Liconix, Santa Clara, CA) for spectra of the CO-bound adducts and 363.8 nm excitation from an argon ion laser (Spectra-Physics, Beamlok 2080) for spectra of the ferric form of the enzyme to enhance the Fe–Cys stretching mode. The
incident laser power on the sample was kept under 3 mW. The cylindrical sample cell was rotated at ~6000 rpm during the spectral acquisition to avoid photodamage to the sample. The scattered light was collected and focused onto an entrance slit (100 μm) of a 1.25 m SPEX spectrophotometer (Jobin Yvon, Edison, NJ) and subsequently detected with a liquid nitrogen-cooled CCD (Roper Scientific, Princeton, NJ). All of the resonance Raman spectra were frequency-calibrated by using spectral lines from indene, except for those in the 1800–2000 cm⁻¹ spectral region, where an acetone/ferricyanide combination was used instead. Cosmic ray artifacts were removed from the spectra by using a routine in the Winspec spectral acquisition software (Roper Scientific). Most of the data were integrated for ~30 min. Longer integration times of 180 and 360 min were used to improve the signal-to-noise ratio for the Fe–Cys and C–O stretching frequency regions. All measurements were taken at room temperature. Optical absorption spectra were obtained before and after each resonance Raman measurement to ensure the integrity of the enzyme.

3.3 RESULTS AND DISCUSSION

GsNOS (Geobacillus stearothermophilus Nitric Oxide Synthase) is a thermostable bacterial NOS that forms a highly stable heme-oxygen complex [18]. This protein, which is homologous to mammalian NOS enzymes, has also served as a useful model for exploring the NOS catalytic mechanism, which involves the oxidation of L-arginine to nitric oxide [19]. Previous over-expression of heme proteins in our lab has been aided by the addition of the heme precursor δ-ALA to the growth media at the time of induction; this procedure resulted in complete heme incorporation for NOS proteins from B. subtilis [20] and D.
radiodurans [21]. In what follows, we show that over-expressed and purified gsNOS obtained from E. coli consists of two species: Native gsNOS with heme incorporated into the protein and gsNOS with protoporphyrin IX (free-base porphyrin, heme without Fe inserted) bound instead of heme. We hypothesize that in over-expressed heme proteins from E. coli that show partial heme incorporation, a substantial population of protein also has protoporphyrin IX bound. Co-expression of just one protein, FC, along with addition of a small amount of δ-ALA is sufficient to rectify this problem.

3.3.1 UV-Vis spectroscopy and SDS-PAGE analysis of gsNOS

Heme and free-base porphyrin have strong π-to-π* transitions (Soret peaks) in the 390-430 nm region of the UV-Vis spectrum. A UV-Vis spectroscopic analysis of gsNOS over-expressed by itself shows that the amount of heme (or porphyrin) incorporated with the protein changes with each batch. The ratio of Soret peak height (403 nm, for high-spin gsNOS) to protein peak height (280 nm) (Abs_{403} / Abs_{280}) varies between 0.25 – 0.40. Co-Expression of FC with gsNOS increases Abs_{403} / Abs_{280} to a maximum value of 0.6 (Figure 3.1). This value remains constant for different protein preparations. This suggests that gsNOS co-expressed with Ferrochelatase dramatically increases heme content of the protein. We also tested if foregoing the addition of δ-ALA, while over-expressing FC, would also result in complete heme incorporation. The purified protein checked for total heme-incorporation either by SDS-PAGE (see below) or by measuring a UV-Vis spectrum showed that although the protein had a heme content that was higher than when δ-ALA was added without FC co-expression, the sample did not have complete heme-incorporation (Abs_{403} / Abs_{280} ratio ~0.5). Addition of a small amount of δ-ALA
(10 mg/L) is sufficient to make up for the slow rate of δ-ALA biosynthesis and allow for complete heme loading.

![UV-Vis spectra](image)

**Figure 3.1:** UV-Vis spectra of gsNOS expressed by itself (thin line) and gsNOS expressed with Ferrochelatase (thick line). Co-expression of Ferrochelatase results in a significant increase in heme content of gsNOS ($\text{Abs}_{403}/\text{Abs}_{280}$). This value saturates at 0.6, indicating almost complete heme incorporation. Inset: GsNOS expressed by itself results in two bands (lane A), both of which shift on His-tag cleavage (lane B). GsNOS co-expressed with Ferrochelatase results in just one band (lane C) that shifts on His-tag cleavage (lane D) as expected.

Purified gsNOS, when over-expressed in *E. coli* with 25 mg/L δ-ALA added at the time of induction, results in two bands both close to 44 KDa on SDS-PAGE (Figure 3.1, Inset, lane A). Both bands shift on His-tag cleavage with thrombin (Figure 3.1, Inset, lane B), indicating that both bands derive from gsNOS with different mobility characteristics in the gel. Mass spectrometry of the protein sample does not indicate any proteolysis and shows a sharp peak for the full-length protein at 44.2 KDa. This suggested that the apparent difference in molecular weight on the gel is due to species that differ in their mobility not because of proteolysis, but probably because of a net charge difference. SDS-
PAGE normally denatures proteins and dissociates them from their cofactors. However, in the case of Cys-ligated hemes, the Fe-S-cys bond can be strong enough to retain the cofactor in the denatured state. Moreover, thermostable proteins can retain some native structure and their cofactors even in the presence of detergent. We suspected that the mobility difference between the two GsNOS species resulted from two populations that differed in their cofactor content (see below). Protoporphyrin IX is the penultimate product in the heme biosynthesis pathway. Ferrochelatase catalyzes the last step in heme biosynthesis i.e insertion of an iron atom into protoporphyrin IX, producing heme as the final product. GsNOS, when co-expressed with FC from E. coli BL21 (DE3) cells, results in only a single species (corresponding to the lower band of the two observed before), as observed by SDS-PAGE (Figure 3.1, Inset, lane C), which on thrombin cleavage shifts as would be expected from the loss of the His-tag peptide (Figure 3.1, Inset, lane D).

3.3.2 Resonance Raman and Fluorescence Analysis of gsNOS

The first clue as to the identity of the additional gsNOS species on SDS-PAGE came from the Resonance Raman studies of gsNOS in the presence of substrate L-arginine. A fresh sample of gsNOS (with 2 mM L-arginine) shows vibrational frequencies at 662 cm⁻¹, 738 cm⁻¹, 783 cm⁻¹, 1360 cm⁻¹ and 1543 cm⁻¹, in addition to the typical vibrational frequencies that have been previously reported [22,23] for other NOSs (Figure 3.2b). These bands disappear after exposure to laser (Figure 3.2c) and thus the difference spectrum (before and after laser exposure) highlights their presence (Figure 3.2d).
Figure 3.2: The Raman spectra of gsNOS from gsNOS co-expressed with ferrochelatase (a), gsNOS expressed by itself (b) and (c). The spectrum in (b) is obtained with 3 mW of 413.1 nm laser excitation with an acquisition time of 5 min. The spectrum in (c) is for the same sample as (b), but obtained after prolonged irradiation with 42 mW of 413.1 nm laser for two hours. (d) shows the difference spectrum (b) – (c), highlighting spectral lines resulting from the photosensitive protoporphyrin IX contamination. The fluorescence spectrum in (e) obtained after a 397 nm excitation is representative of that of protoporphyrin IX bound to protein (see text) and is from the same sample in (b).

The frequencies apparent in the difference spectrum are typical of free base porphyrin, which is known to be especially photo-sensitive [24,25]. When gsNOS was co-expressed with FC, and the media supplemented with δ-ALA (10 mg/L of cell culture or 60 μM, ~ $0.50 per liter) the vibrational bands from porphyrin were completely absent (Figure 3.2a). Furthermore, the fluorescence spectrum of gsNOS (Figure 3.2e) measured with excitation at 397 nm is characteristic of the fluorescence spectrum for free-base porphyrin [26]. This is not observed in the gsNOS-FC sample. All these results indicate that a substantial population of the protein when over-expressed alone has free-base porphyrin bound, whereas the rest of the protein has covalently bound heme. Co-expression of ferrochelatase allows for complete heme incorporation. Our
data suggests that the last step of heme biosynthesis i.e Fe insertion into porphyrin by FC is the limiting step in recombinant heme protein production in E. coli when excess d-ALA is provided.

3.3.3 Complete heme incorporation assisted by Ferrochelatase in a bacterial P450 (BP450) and a heme binding PAS domain containing protein (HBPAS)

In addition to gsNOS, FC also increases heme content to saturating levels in two other heme proteins: 1) BP450, a Cys-ligated heme protein and 2) HBPAS, a His-ligated heme protein. Both of these proteins, when over-expressed in E. coli result in proteins with under-incorporated heme. BP450, like gsNOS is a Cys-ligated heme protein. UV-Vis spectra of BP450 co-expressed with FC gives much more pronounced Soret peaks than when expressed alone (Figure 3.3). When analyzed by SDS-PAGE, BP450 also shows two closely spaced bands (Figure 3.3, Inset, lane A), which both shift on His-tag cleavage (Figure 3.3, Inset, lane B). Like gsNOS, co-expression with FC (Figure 3.3, Inset, lane C) generates only one band (Figure 3.3, Inset, lane D). HBPAS expressed alone, always results in a single band on a SDS-PAGE gel (not shown), but the protein shows absorption for four Q-bands in the UV-Vis spectrum (Figure 3.4). Heme proteins usually show two such bands. The extra band are representative of protein bound to protoporphyrin IX [26]. Co-expression of Ferrochelatase results in an increase in the $\text{Abs}_{(\text{Soret})}/\text{Abs}_{(280)}$ ratio, which indicates increased heme incorporation, and the Q-bands associated with free porphyrin disappear (Figure 3.4). The fluorescence spectrum of HBPAS without co-expression of FC is similar to that of gsNOS expressed without FC in that it indicates the presence of protoporphyrin IX. Protein co-expressed with FC shows no free porphyrin fluorescence.
Figure 3.3: UV-Vis spectra of BP450 expressed by itself (—) and BP450 expressed with ferrochelatase (—). Co-expression of ferrochelatase results in a substantial increase in heme content of BP450 ($\text{Abs}_{580}/\text{Abs}_{680}$). Inset: BP450 expressed by itself also results in two bands (lane A) just like gSNSOs, both of which shift on His-tag cleavage (lane B). BP450 co-expressed with Ferrochelatase results in one band (lane C), which shifts on His-tag cleavage as expected (lane D).

Figure 3.4: A Heme Binding PAS domain containing protein (HBPAS; His-ligated) when expressed by itself (—) shows four peaks Q-band absorption (inset). Co-expression with ferrochelatase (—) increases the heme content and results in only two Q-bands. Fluorescence measurement also indicates that the protoporphyrin contamination issue is solved by Ferrochelatase co-expression.
3.4 CONCLUSIONS

δ-ALA production is the rate-limiting step in heme biosynthesis[27-30] and δ-ALA synthesis is inhibited by heme. The results given here suggest that when δ-ALA is supplied externally, heme incorporation into an over-expressed protein depends on the insertion of Fe into porphyrin. Notably, the proteins tested here fold into their native structures with free base porphyrin. The method of co-expressing FC and supplementing with d-ALA is effective for both Cys-ligated and His-ligated proteins. In case of a Cys-ligated protein, this deficiency can be observed on an SDS-PAGE gel, whereas, UV-Vis spectra / Fluorescence measurements are needed to detect the presence of free prophyrin in His-ligated proteins. A greater covalent character for the Cys S-Fe bond might be the reason why proteins with and without Fe migrate differently on a gel. The heme likely remains bound to the protein through the Cys-Fe ligation, whereas the free porphyrin is lost. For a His N-Fe ligation, the interaction is probably not strong enough to withstand denaturation and the heme is lost from the protein irrespective of the heme content of the sample. In summary the relatively simple and inexpensive method described here may be useful for over-expressing many different proteins with full heme content in *E. coli*. 
REFERENCES


CHAPTER 4

ALTERNATIVE X-RAY CRYSTAL STRUCTURES OF A BACTERIAL NITRIC OXIDE SYNTHASE: ENHANCING CRYSTAL CONTACTS BY SURFACE ENTROPY REDUCTION

4.1 INTRODUCTION

Nitric oxide synthases (NOSs) from animals are highly regulated enzymes that catalyze the oxidation of L-arginine (L-arg) to nitric oxide (NO)\(^1\). Homologous enzymes are found in several bacteria, mostly gram positive. For example, *Geobacillus stearothermophilus*, a thermophilic bacterium, contains a NOS (gsNOS) that forms a highly stable oxy-heme complex with a lifetime that is 10-100 times longer (at 4 °C) than those of other NOSs \(^2\). This characteristic makes gsNOS attractive for biochemical and structural studies aimed at elucidating aspects of the NO biosynthesis mechanism \(^3\). In particular, the highly reactive heme-oxy species responsible for substrate oxidation have been difficult to characterize \(^4\). X-ray crystallography would offer a direct view of the heme-oxy intermediates, given that these species could be trapped in crystals. However, for such structural analyses to be useful and provide information on the oxygen geometry relative to substrate and the details of the hydrogen bonding networks within the active center, the resolution of the X-ray structures would likely need to exceed 2.2 Å. We have previously reported a 3.2 Å resolution structure of gsNOS, which reveals some interesting features of the active center potentially related to the ability of this enzyme to stabilize ligated heme states \(^2\). Nonetheless, crystals that diffract
to higher resolution are needed to pursue additional structures aimed at informing on catalytic mechanism. Surface entropy reduction (SER) of protein surfaces by substituting surface residues (Lys, Glu, Gln) with high degrees of rotational freedom with residues containing side-chains with lower potential conformational entropy (Ala, Tyr, Ser), and favored at protein-protein interfaces is becoming a popular technique to enhance lattice contacts and improve crystals (5-24). Herein, we report two new crystal forms of gsNOS, obtained as a result of the SER method, that diffract to much higher resolution than the original crystals. We also show that the residue substitutions participate intimately in the crystal contacts. Unfortunately these crystals did not prove useful for mechanistic studies because the gsNOS structure is substantially perturbed in each case. Thus, this work reaffirms that efficacy of the SER method for finding new crystal forms, but raises a cautionary flag with respect to the interpretation of structures garnered from modified proteins crystallized at non-physiological conditions.

4.2 EXPERIMENTAL PROCEDURES

4.2.1 Materials: Sodium chloride, sodium acetate, magnesium chloride and Isopropanol were obtained from Mallinckrodt. TRIS was from JTBaker, and L-arginine was from Fluka.

4.2.2 Cloning and expression of gsNOS variants: The various amino substitutions were generated in gsNOS by using the QuickChange mutagenesis kit from Stratagene. The proteins were expressed and purified in a manner identical to that carried out for native gsNOS (2).
4.2.3 Crystallization, Data collection and Model Building: Single crystals of
diffraction quality were grown by vapor diffusion from a solution that
contained 1 mM protein, 2 mM L-arginine, 50 mM TRIS pH 7.5 and 150 mM
NaCl mixed 1:1 with reservoir solutions from a set of crystal screens. The
Q36Y-E37Y variant produced a hit after several weeks in Hampton Crystal
Screen 2, condition 20: 0.1 M MES pH 6.5, 1.6 M MgSO₄·7H₂O. The optimized
condition for this variant, 0.1M TRIS pH 8.5 and 1.2 M MgSO₄ resulted in 200
μM X 200 μM X 400 μM crystals that grew over 2-7 days. The crystals belonged
to the P₃₂₁ space group with cell dimensions 165.5 X 165.5 X 136.6 Å and α =
β = 90°, γ = 120°. Diffraction data (2.6 Å) for this variant was collected at
CHESS beamline A1 on a Q4 quantum CCD detector, with the wavelength set
at the Se K-edge. The Q36A-E37A variant produced a hit after several days
from Hampton Crystal Screen, condition 24: 0.2 M CaCl₂·2H₂O, 0.1M sodium
acetate trihydrate pH 4.6, 20% Isopropanol. The optimized condition for this
variant, 2-5% isopropanol, 0.1 M Sodium Acetate pH 3.6-4.0 resulted in 400 μM
X 400 μM X 600 μM crystals that grew in one week. The crystals belonged
to the P₃₂ space group with cell dimensions 90.6 X 90.6 X 111.8 Å and α = β = 90°,
γ = 120°. Diffraction data (2.2 Å) for this variant was collected at a home source
using a Rigaku R-axis++ detector and Cu-Kα radiation from a Rigaku RU-300
rotating anode generator. The wavelength of the radiation used was 1.5418 Å.
Both the datasets were reduced and scaled using HKL2000(25). The 3.2 Å
structure of gsNOS (PDB ID: 2FLQ) was used as a model for molecular
replacement with Phaser(26). The structures were then adjusted with XFIT(27)
and COOT(28) to Fₒ – Fc and 2Fₒ – Fc maps. Addition of L-arginine, heme and
water molecules amid cycles of refinement using CNS(29) produced the final models (Table 4.1)

Table 4.1: Data collection and refinement statistics for gsNOS complexed with L-arginine.

<table>
<thead>
<tr>
<th></th>
<th>Q36Y-E37Y gsNOS</th>
<th>Q36A-E37A gsNOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution (Å)</td>
<td>2.60 (2.74 – 2.60)</td>
<td>2.20 (2.31 – 2.20)</td>
</tr>
<tr>
<td>Ligand, Prosthetic group</td>
<td>L-arginine, Heme</td>
<td>Heme</td>
</tr>
<tr>
<td>Number of residues</td>
<td>709</td>
<td>674</td>
</tr>
<tr>
<td>Number of waters</td>
<td>403</td>
<td>339</td>
</tr>
<tr>
<td>No. of unique reflections</td>
<td>66322</td>
<td>27526</td>
</tr>
<tr>
<td>Number of observations</td>
<td>1369092</td>
<td>940784</td>
</tr>
<tr>
<td>% Completeness</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>$I_{av}$ $^a$</td>
<td>34.8 (3.41)</td>
<td>32.8 (6.35)</td>
</tr>
<tr>
<td>$R_{sym}$ $^b$ (%)</td>
<td>7.4 (62.0) $^g$</td>
<td>6.7 (45.6) $^g$</td>
</tr>
<tr>
<td>$R$ (%)</td>
<td>21.58</td>
<td>24.0</td>
</tr>
<tr>
<td>$R_{free}$ $^d$ (%)</td>
<td>22.89</td>
<td>24.9</td>
</tr>
</tbody>
</table>

$^a$ Intensity of the signal to noise ratio. $^b$ $R_{sym} = \sum_i |I_i - \langle I \rangle_i| / \sum_i |I_i|$. $^c$ $R = \sum |F_{obs} - |F_{calc}| / \sum |F_{obs}|$ for all reflections (no $\sigma$ cutoff). $^d$ $R_{free}$ calculated against 10% of reflections removed at random. $^e$ Overall model average thermal ($B$) factor. $^f$ Root mean square deviations from bond and angle restraints. $^g$ Highest resolution bin for compiling statistics.

4.3 RESULTS AND DISCUSSION

The publicly available SER server (http://nihserver.mbi.ucla.edu/SER/) analyzes protein sequences for potential loop regions that contain a succession of residues with long, generally hydrophilic side chains. For the gsNOS sequence The SER server suggested that the residue stretches E35-Q36-E37, Q185-K186, K19-E21-Q22 and Q320-E321-K322 were sites that could be substituted by alanine prior to crystallization trials. Further analysis of the known 3.2 Å resolution structure of gsNOS indicated that some of the identified residues were structurally important and thus, these were
discounted. Furthermore, additional sites for potential residue substitution were identified by inspection of the structure for clusters of Lys, Gln, Glu and Arg residues on the surface. The resulting five clusters of residues that did not seem important for stabilizing the overall fold of the protein were then substituted to alanine and tyrosine, generating 10 different gsNOS variants, all of which contained 2-3 residue substitutions: Q36A-E37A, Q36Y-E37Y, E96A-E97A-E98A, E96Y-E97Y-E98Y, E145A-E146A, E145Y-E146Y, Q185A-K186A, Q185Y-K186Y, Q319A-Q320A-K322A, Q319Y-Q320Y-K322Y. Out of the 10 variants, only 6 were soluble: all five variants with only Ala substitutions plus the Q36Y-E37Y variant. Of these, only two gave diffraction quality crystals: Q36Y-E37Y and Q36A-E37A.

4.3.1 Crystallographic structure of the Q36Y-E37Y variant

The 2.6 Å resolution structure of dimeric gsNOS Q36Y:E37Y is almost identical to that of the native gsNOS dimer (Cα-RMSD: 0.6 Å for 359 out of 359 residues). The only minor differences are slight re-arrangements of side-chain conformation in regions where substitutions were made to enhance crystal contacts. Electron density for side chains of residues N42 and W47, which are in the vicinity of the mutation site, were not observed in the original gsNOS structure where both were modeled as alanine(2). This new structure contains well-defined electron density for both of these residues. The residues that were substituted by Tyr, Gln36 and Glu37, are at the beginning of an α-helix on the periphery of the molecule (Figure 4.1). Interestingly, the introduced Tyr side chains provide key interactions for the association of two gsNOS dimers in the crystal lattice (Figure 4.1 A). A symmetric α-helix-to-α-helix interaction
forms the basis for the dimer interaction. A zoom-in view of this interaction region is shown in Figure 4.1 B. Interestingly, one of the tyrosine residues closely $\pi$-stacks with a tryptophan, and the other tyrosine interacts with this tryptophan via a hydrogen bond within this interface (Figure 4.1 B).

![Crystal contacts in gsNOS Q36Y-E37Y.](image)

Figure 4.1: Crystal contacts in gsNOS Q36Y-E37Y. (A) Two gsNOS dimers interact in the crystal through the SER substitutions. One significant interaction between the shown molecules in the interaction between the two $\alpha$-helices. (B) The region of the crystal contacts: Y36 of one dimer is involved in $\pi$-stacking with W47 of another dimer. The Y37-OH may make a weak hydrogen bond with W47 indole NH, although the heavy atom separation at 3.9 Å is longer than that of a typical hydrogen bond. Y36 also interacts with R40, which in turn stacks with R'40 across the dimer interface. (C) A model of native gsNOS in the same packing environment shows that without the Tyr substitutions the crystals contact would be disfavored.

If these tyrosine residues were substituted back to their native Glu / Gln residues (Figure 4.1 C), no such stabilizing interaction would be possible and in fact the contact would be disfavored by electrostatics.
4.3.2 Crystallographic structure of the Q36A-E37A variant

The 2.2 Å structure of gsNOS Q36A:E37A differs from that of native gsNOS primarily in the arrangement of the dimer. The structure of the subunits alone are quite similar when comparing the variant to the native (Cα-RMSD: 0.78 Å for 335 out of 359 residues), but the variant has 24 residues per subunit that are disordered and not visible in the electron density map.

Figure 4.2: Crystal contacts in Q36:E37A. (A) Two gsNOS dimers interact in the crystal through the SER substitutions. (B) A37 is very close to E146, and any residue with a larger side chain would cause a steric clash. (C) A model of the same structure, with the two alanine residues changed to native gsNOS residues, showing that native gsNOS in such an orientation would cause a steric as well as a charge repulsion interaction, making such orientation of two dimers unstable.

In the regions that the substitutions were made to enhance crystal contacts, there are minor variations compared to native. Once again, the SER
substitutions again mediate an important contact in the crystal lattice (Figure 4.2 A). Ala36 and Ala37 associate with a 4-residue \( \alpha \)-helix from another dimer that contains E146. The Ala residues overcome unfavorable steric and electrostatics to allow the contact with E146 (Figure 4.2 B, 4.2 C). We hypothesize that other substitutions at residue 37, which reduce the size and negative charge of the side chain, would facilitate a similar interaction. Furthermore, the structure suggests that Q36 may not have to be substituted to get this crystal form of gsNOS. Notably, 2.2 Å resolution diffraction data was collected from gsNOS Q36A:E37A crystals on a rotating anode; considerably higher resolution is reasonably expected with a synchrotron source. However, synchrotron data was not pursued for this variant due to the reasons outlined below.

4.3.3 Active site structures of the SER variants

Crystallization of both gsNOS SER variants (Q36Y-E37Y and Q36A-E37A) was attempted in the presence of 2 mM L-arginine, which is a two-fold excess relative to total protein and also greater than the binding constant (2). The active sites of both variants were expected to be similar to that of native gsNOS, which possessed a slightly more compact active site structure when compared to other bacterial NOSs(30, 31) and mammalian NOSs(32) that have L-arg bound (not shown). The structure of the Q36Y-E37Y variant reveals an active site that is similar to the other NOSs and without the minor variations that characterized the the native gsNOS structure (2). These structural differences primarily involved an active site Ile223 residue that resides above the open heme iron coordination site. In native gsNOS the side chain of this
residue shifts 0.6 Å closer to the heme iron than in other NOSs. This shift in Ile223 was correlated with natural residue substitutions in gsNOS that appeared to affect the conformation of the Ile223 peptide backbone. No such effect on Ile223 is observed in gsNOS Q36Y:E37Y, despite the backbone interactions being preserved. L-Arg is bound in a mode very similar to that of native gsNOS. Since we had observed a more compact alternate conformation for the active site in the native gsNOS structure, with well-defined electron density (at 3.2 Å resolution), the data suggest that these two might be alternating conformations that could be in equilibrium.

Figure 4.3: A comparison of the active sites of gsNOS at pH 8.5 versus pH 3.6. At pH 8.5 (yellow), E248 participates in substrate binding by forming two hydrogen bonds with the L-Arg guanidinium group. At pH 3.6 (dark orange), E248 faces the solvent channel and hydrogen bonds with the heme carboxylate. The latter interaction indicates that either Glu248 or the heme carboxylate or both are protonated and low pH.

In contrast, the active site conformation of the 2.2 Å resolution Q36A-E37A structure is drastically different from that of native, probably because of the low pH (3.6) of the reservoir solution, which is mixed with the protein solution to form crystals. In L-arginine bound structures of NOSs, a conserved glutamate residue, E248, lies in the active site and forms several charged
hydrogen bonds with bound substrate (Figure 4.3) (33). In the Q36A:E37A structure, E248 is directed away from the substrate binding site and instead hydrogen bonds to one of the heme carboxylate groups. This interaction is probably promoted by the low pH of the crystals (3.6-4.0). As a result no Arg binds in the active center and the pH is too low to support catalytic activity.

4.3.4 Dimeric forms of gsNOS

Figure 4.4: Overall structure of gsNOS at pH 8.5 versus pH 3.6. (A) The structure gsNOS variant Q36Y-E37Y at pH 8.5 is almost identical to most other known NOS structures, with a normal Heme-Heme distance of 34.5 Å. (B) The structure of gsNOS variant Q36A-E37A at pH 3.6 shows loss of structured elements in the dimer interface, particularly the region known as the helical lariat that binds the pterin cofactor. This results in a more compact structure, with a Fe-to-Fe distance of 30.5 Å.

The overall structure of the Q36Y-E37Y variant (Figure 4.4 A) is almost identical to the native gsNOS, with a well-defined dimer interface and a normal distance of ~34.5 Å between the two hemes (specifically the Fe atoms). However, the overall structure of the Q36A-E37A variant dimer is drastically different. Most of the residues in the dimer interface (residues 330 – 355) are completely absent in this structure and the dimer is "compressed" (Fe-to-Fe
distance of 30.5 Å), with the two subunits closer together than in typical NOS structures (Fe-to-Fe distance of 34.5 Å) (Figure 4.4 B). Due to the disordered dimer interface, the active site is more solvent exposed, which may facilitate protonation of the E248, and thereby disruption of substrate binding.

4.4 CONCLUSIONS

The SER methodology was used to obtain high-resolution crystal structures of gsNOS, for which only a low-resolution structure was previously available. This method may well be effective with other proteins where initial crystallization attempts fail as our results indicate that the methods indeed generates surface features that facilitate crystal lattice contacts. However, the main goal of our study was to obtain high-resolution crystal forms of gsNOS that could be then used to trap the oxy-heme complexes in the crystal for mechanistic studies. The following are characteristics of a crystal form that would be necessary for such studies: (a) high resolution diffraction to define the geometry of an oxygen molecule bound to heme and details of hydrogen bonding in the active center; (b) crystal growth or tolerance at physiological pH, as the oxygen complex is sensitive to pH and higher pH enhances its stability; (c) stability of the crystals to chemical reduction; (d) stability of the crystals to lower temperatures (i.e. -10 °C) and cryoprotectants. The current gsNOS crystals do not meet all these requirements but the generation of additional variants by SER offers promise of new crystals forms that will be suitable.
REFERENCES


CHAPTER 5

EPR AND ENDOR CHARACTERIZATION OF INTERMEDIATES IN THE REACTIONS OF CRYOREDUCTED OXY-NITRIC OXIDE SYNTHASE FROM G. STEAROTHERMOPHILUS WITH BOUND SUBSTRATES

5.1 INTRODUCTION

Nitric oxide (NO) plays essential role in biological processes occurring in mammals such as vasodilation, neurotransmission, and immune responses (1, 2). In eukaryotes, NO is mainly synthesized by nitric oxide synthase (NOS) (3). NOS catalyses the O₂ dependent conversion of L-arginine (Arg) to L-citrulline (Cit) and NO via two consecutive reactions that generates N⁰-hydroxy-L-arginine (NOHA) as a stable intermediate (4). Mechanistic studies suggest that catalysis by all the three isoforms of mammalian NOSs (mNOSs) involves two successive mono-oxygenation reactions, the first a Ferryl-heme dependent hydroxylation of a guanidino nitrogen of Arg yielding NOHA and the second, a nucleophilic attack by peroxy heme on the guanidino carbon of NOHA (5). mNOSs are homodimeric enzymes that contain a reductase domain with binding sites for FAD, FMN and NADPH and an oxygenase domain that binds heme, substrate and the cofactor tetrahydrobiopterin (BH4). During catalysis, mediated by calcium/calmodulin, electrons transfer from NADPH through FAD and FMN in the reductase domain of one subunit of the homodimer to the oxygenase domain of the other subunit (6). The crystal structures of the oxygenase domains of mammal NOSs show that the heme is coordinated by a cysteine residue on the proximal side as in
cytochromes P450 and substrates Arg and NOHA bind above the heme iron atom in the distal pocket (7, 8). The BH4 binding site is in the vicinity of the heme propionates (7). In the resting state of NOS the heme is primarily six-coordinate having water as 6-th axial ligand. Substrate binding converts the heme center to 5-coordinate high-spin S=5/2 configuration.

![Diagram of the NOS reaction cycle](image)

**Figure 5.1: The NOS reaction cycle**

The NOS reaction sequence is well understood (Figure 5.1), although the nature of the heme-oxygen complexes directly involved in substrate oxidation remains largely unknown. In both Arg and NOHA reactions, BH4 bound ferric NOS is first reduced to the ferrous state by NADPH reductase domain. It is followed by binding of O2 to form the oxy complex spectroscopic properties of that are modulated by the substrate binding (9). In substrate free and substrate bound oxy NOS heme oxy species have been interpreted as the ferrous-dioxygen and the ferric-superoxy-complex, respectively (10-12). BH4 reduces ferric-superoxy species in both steps of NO synthesis to form peroxo ferric or hydroperoxy ferric NOS intermediates. The BH4 radical formed in first step is reduced by the reductase domain while in NOHA reaction the BH4 radical is
reduced probably by forming NO or Fe(III)NO complex (5). There is general consensus that conversion of L-arginine to NOHA likely proceeds as a cytochrome P450-type mono-oxygenation (13) with the high-valent iron-oxo species as a catalytically active intermediate. A hydroperoxy- or peroxy species is proposed to act in conversion NOHA to Cit and NO. However, it was also shown that mNOSs (14, 15) catalyze oxidation of NOHA to Cit, cyano-ornithine and HNO in the presence of H₂O₂. In addition, some resin supported Fe(III) porphyrins are efficient catalysts for H₂O₂ mediated oxidation of Arg to Cit and NO (16). These observations thus do not allow for complete elimination of the ferryl species as a catalytically active intermediate in NOHA oxidation.

Since the decay of the primary oxy-NOS complex occurs slower than the decays of the subsequent intermediates, none of the latter intermediates have been detected during a single turnover reaction under physiological conditions. Application of cryo-reduction in combination with advanced EPR spectroscopy enables trapping and characterization of active oxy species that form during reaction of one-electron reduced oxy-NOS with substrate (17). Radiolytic reduction of oxy-endothelial-NOS heme domain (oxy-eNOS) with bound Arg or NOHA in the presence of 4-amino tetrahydrobiopterin at 77K yields peroxy-Fe(III) NOS intermediate as the primary product. The conversion of peroxy-Fe(III) NOS Arg complex to product-state intermediate occurs at relatively low temperature of 165-170K. EPR and proton ENDOR spectra of the intermediate formed with Arg as substrate support the suggestion that the reaction involves the formation and attack of Compound I (17). However at no stage of reaction/annealing does one observe an EPR signal from a hydroperoxy-Fe(III) state with either substrate which is in sharp
contrast to the other heme enzymes like cytochromes P450 (18). Low stability of oxy-e-NOS complexes at high protein concentrations makes it difficult to carry out more detailed studies with mNOSs. We recently reported that a NOS homolog from the thermophilic bacterium *G. stearothermophilus* (gsNOS) forms an oxy-complex that is much more stable than the ones formed by mNOSs or other bacterial NOSs (bNOSs) (19). The bacterial NOS-like proteins are similar to oxygenase domain of mNOSs and contain no associated reductase domain (19-21). NOSs from *B. subtilis* and *D. radiodurans* have been shown to accept electrons from mammalian reductase domains and produce NO (22, 23). BH₄ acts as electron donor to the oxy-gsNOS species in both steps of NO synthesis (19). The crystal structure of gsNOS is very similar to that of the oxygenase domain (NOSoxy) of mammalian NOS including the substrate-binding site. Like mammalian NOS, Soret band of oxy-Fe(II)gsNOS complex shifts from 413 nm to 427 nm in the presence of substrates. The substrate-induced red shift correlates with hydrogen bonding interactions between substrate and heme bound dioxygen resulting in conversion to ferric heme superoxy species (9, 10, 19).

5.2 EXPERIMENTAL PROCEDURES

GsNOS was expressed and purified as before (19). After this, the concentrated protein was diluted 10 times into 100 mM Tris pH 8.5, 150 mM NaCl, 50% Ethylene Glycol (EG) and concentrated back to 1 mM. When needed, the protein was exchanged into buffers made using D₂O and D₂-EG. Also, when needed, Arg, NOHA were added in equimolar amounts and Me-Arg, NO₂-Arg or Cit were added in 5-fold excess to ensure full complex formation. All the Fe(II)-gsNOS samples were made by reducing Fe(III)-
gsNOS exactly using equivalent amounts of Dithionite inside an anaerobic glove box. The samples were then frozen in liquid nitrogen. These samples were then warmed up to ~20 °C and oxygen gas was bubbled and the samples stored in quartz EPR tubes at 77 K until cryo-reduction. The oxy-gsNOS samples were reduced at 77 K by γ-irradiation with a 60Co source to a dose of ~3 Mrad and then kept at 77 K. In general, the samples were examined by EPR within 12-24 h, and returned to 77 K storage for later annealing and EPR and ENDOR study. As desired, the cryo-reduced samples were annealed at temperatures above 77 K by transferring a sample from liquid nitrogen to a bath at fixed temperature for 1 min unless specified otherwise, after which the sample was transferred back to liquid nitrogen. For consistency, we quote clock times for annealing. However, examination of a thermocouple frozen in a Q band tube showed that an approximate equilibrium with the bath occurred within 15-20 s. Thus, a reported 1 min annealing corresponds to 40-45 s at each temperature. X-band spectra were recorded on a modified Varian E-4 spectrometer at 77 K. Q-band (35 GHz) EPR and ENDOR spectra were recorded on a Varian E-110 spectrometer equipped with a helium immersion Dewar.

For product identification, 6M Urea (final) was added to the protein solution to dislodge the products and the amino acids were then derivatized using O-phthalaldehyde as described and the products were then analyzed using HPLC. The samples were run through an HP ODS Hypersil 5-μm 100X21 mm C18 column. The derivatized samples were detected by fluorescence (Excitation: 360 nm, Fluorescence: 465 nm). Elution times for Arg,
NOHA and Cit were determined using standards, and were compared to products of the cryo-reduction and annealing reactions.

5.3 RESULTS

5.3.1 Effects of substrates and inhibitors on heme site of gsNOS

5.3.1.1 Ferric gsNOS: We have studied the effect of the substrates and products as well as some inhibitors on the EPR spectra of ferric gsNOS to characterize their binding to the protein, to generate reference spectra for assignment of ferric species in the EPR spectra of the irradiated samples of the oxy-gsNOS complexes, to help in identification of product complexes formed during cryo-reduction at step-annealing and to compare EPR properties of ferric gsNOS complexes with those reported for mNOSs. Resting state, substrate free Fe(III) gsNOS exists preferably as a low-spin aqua-Fe(III)heme form showing a dominant rhombic EPR signal with \( g=\{2.42, 2.28, 1.92\} \) and a minor signal with \( g=\{2.51, 2.29, 1.88\} \) (Table 5.1, Figure 5.2). Relative contribution of the latter significantly increases in ferric state of gsNOS formed during auto-oxidation of oxy-gsNOS–NOHA complex at \(-25^\circ\text{C}\) for 5 min (Figure 5.2). Axial water ligand coordinated to heme Fe(III) shows strongly coupled proton ENDOR signal with \( A_{\text{max}}\approx 10\) MHz (Figure 5.3). \( g\)-Tensor components for the minor low-spin ferric species showing more rhombic EPR signals are similar to those reported for alkaline forms of ferric cytochrome P450 and CPO (24), suggesting that a hydroxide ion (OH\(^-\)) is coordinated to Fe(III) heme. This supposition is also supported by observation of strongly coupled exchangeable H ENDOR signal at \( g_1=2.51 \) with \( A=13\) MHz (not shown). Addition of Arg, NOHA as well as Arg analogs / inhibitors Me–N\(^w\)–Arg and NO\(_2\)–N\(^w\)–Arg results in conversion of an overwhelming majority of the
enzyme to the five coordinate, high-spin substrate bound form with characteristic rhombic EPR signals, g-tensors of which are presented in Table 5.1. g-Tensor components depend noticeably on the structure of the substrates.

Table 5.1: g-Tensor components for ferric gsNOS complexes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Species</th>
<th>( g_1 )</th>
<th>( g_2 )</th>
<th>( g_3 )</th>
</tr>
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<tbody>
<tr>
<td>Fe(III)gsNOS</td>
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<td>LS (major)</td>
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<td>2.28</td>
<td>1.916</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LS (minor)</td>
<td>2.49</td>
<td>2.28</td>
<td>1.87</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HS (minor)</td>
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<td>4.04</td>
<td>1.79</td>
</tr>
<tr>
<td>Fe(III)gsNOS</td>
<td>NOHA</td>
<td>LS</td>
<td>2.42</td>
<td>2.28</td>
<td>1.915</td>
</tr>
<tr>
<td></td>
<td>(auto-oxidation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe(III)gsNOS</td>
<td>Arg</td>
<td>LS</td>
<td>2.51</td>
<td>2.28</td>
<td>1.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HS (major)</td>
<td>7.78</td>
<td>4.0</td>
<td>1.79</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LS</td>
<td>2.43</td>
<td>2.286</td>
<td>1.912</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LS</td>
<td>2.49</td>
<td>2.28</td>
<td>1.87</td>
</tr>
<tr>
<td>Fe(III)gsNOS</td>
<td>NOHA</td>
<td>HS</td>
<td>7.85</td>
<td>3.92</td>
<td>1.77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LS</td>
<td>2.427</td>
<td>2.28</td>
<td>1.912</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LS</td>
<td>2.51</td>
<td>2.28</td>
<td>1.884</td>
</tr>
<tr>
<td>Fe(III)gsNOS</td>
<td>Me-Arg</td>
<td>HS</td>
<td>7.85</td>
<td>3.93</td>
<td>1.77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LS</td>
<td>2.42</td>
<td>2.28</td>
<td>1.915</td>
</tr>
<tr>
<td>Fe(III)gsNOS</td>
<td>NO₂ Arg</td>
<td>HS</td>
<td>7.55</td>
<td>4.27</td>
<td>1.84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LS</td>
<td>2.43</td>
<td>2.28</td>
<td>1.915</td>
</tr>
<tr>
<td>Fe(III)gsNOS</td>
<td>Cit</td>
<td>LS</td>
<td>2.51</td>
<td>2.29</td>
<td>1.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LS</td>
<td>2.42</td>
<td>2.29</td>
<td>1.914</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HS</td>
<td>7.75</td>
<td>4.06</td>
<td>1.794</td>
</tr>
</tbody>
</table>

These results are qualitatively quite similar to those reported for e-NOS (25, 26). Perturbations in the heme center caused by the bound arginine derivatives depend on the chemical structure of the substituents. This might be explained
by the fact that the substituents affect the interaction of the bound arginine with the distal side of heme pocket as observed by Poulos and coworkers for N-alkyl-N'-hydroxyguanidine (27, 28) and by that, modulate the heme environment, including its interaction the cysteine ligand (25). We have not observed similar effects of BH₄ on spectroscopic properties of ferric gsNOS.

Figure 5.2: X-band EPR spectra for substrate free ferric gsNOS in 50% EG-buffer pH 8.5 (A) and ferric gsNOS formed during autooxidation of ternary oxy gsNOS+NOHA complex in 50% EG-buffer pH 8.5 (B) at -25 C for 5 min. Instrument: 28K, AM=10G, 10mW, 9.375 GHz

Figure 5.3: Field dependence of H ENDOR spectra for the low spin aqua ferric gsNOS in 50% EG-buffer pH 8.5. Conditions: T=2K, 35 GHz, AM=1G, rf sweep rate of 0.5MHz/sec, RF power 5W, 15scans.
Addition of Cit to Fe(III)-gsNOS results in a small increase in the high-spin state and the appearance of a new low-spin form with g-values of 2.51, 2.29 and 1.87 (Table 5.1). EPR and HENDOR properties of the new low spin form are very similar to these for the mentioned above, the g_i 2.51 low spin intermediate appearing during low temperature auto-oxidation of ternary oxy-gsNOS-NOHA complex. This similarity of the spectroscopic properties suggests that the low-spin state of citrulline bound NOS iron(III) is coordinated by hydroxide rather then Cit. The low-spin ferric state with similar spectroscopic properties was reported for n-NOS in the presence of Cit (26).

These observations show that EPR properties of ferric gsNOS and its complexes with Arg and its analogs are very similar to these for mNOSs.

5.3.1.2 Ferrous gsNOS. Binding substrates to high-spin penta-coordinate ferrous NOS was shown to have little effect on absorption and RR spectra of heme (11, 12). Recently we have showed that EPR silent Fe(II) hemoproteins can be radiolytically reduced in frozen solution at 77K to EPR active low-valent states (29). The cryo-trapped low-valent iron species retain conformations of the ferrous precursors and may be used as a sensitive EPR probe for testing effect of substrates and inhibitors on the Fe(II) heme center. Figure 5.4 represents typical EPR spectra of ferrous NOS exposed to γ-irradiation at 77K. The spectra disclose three different EPR signals. A rhombic EPR signal with g-values of [2.29, 2.22, 1.94] can be assigned to cryo-generated Fe(I) species (Figure 5.4, Table 5.2). The second rhombic EPR signal with g-values of 4.36, 3.86 and below 2.0 (Table 5.2) was shown to be due to
cryo-generated Fe(II) porphyrin anion radical center with antiferromagnetically coupled Fe(II) ($S=2$) and porphyrin radical ($S=1/2$) (29). The third signal with $g$ values of 7.72, 4.02 and $\sim 1.79$ is due to cryo-generated Fe(III)gsNOS species (29). The presence of two cryo-generated low-valent iron species in cryo-reduced gsNOS indicates that the ferrous precursor had at least two different conformational sub-states. Following the data presented in Table 5.2, Arg, NOHA as well as BH4 affect $g$ values of cryo-generated Fe(I) species indicating that the heme center of gsNOS is perturbed by binding of substrates and BH4. EPR properties of cryo-generated Fe(II) porphyrin anion radical species are only slightly modulated by substrate binding (Table 5.2). Mechanism of perturbing effect of bound substrate on the heme site in Fe(II) NOS is likely the same as that considered above for ferric NOS.
### Table 5.2: g-values for cryo-reduced Fe(II) gsNOS

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Species</th>
<th>g₁</th>
<th>g₂</th>
<th>g₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe(II)gsNOS</td>
<td>None</td>
<td>Fe(I)</td>
<td>2.29</td>
<td>2.22</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fe(II)P</td>
<td>4.36</td>
<td>3.87</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Fe(II)gsNOS</td>
<td>Arg</td>
<td>Fe(I)</td>
<td>2.27</td>
<td>2.21</td>
<td>ND</td>
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<td></td>
<td></td>
<td>Fe(II)P</td>
<td>4.37</td>
<td>3.86</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Fe(II)gsNOS</td>
<td>Arg+BH₄</td>
<td>Fe(I)</td>
<td>2.25</td>
<td>2.20</td>
<td>1.946</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fe(II)P</td>
<td>4.37</td>
<td>3.86</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Fe(II)gsNOS</td>
<td>NOHA</td>
<td>Fe(I)P</td>
<td>2.28</td>
<td>2.23</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fe(I)P</td>
<td>2.30</td>
<td>2.26</td>
<td>ND</td>
</tr>
<tr>
<td>Fe(II)gsNOS</td>
<td>NOHA</td>
<td>Fe(II)P</td>
<td>4.37</td>
<td>3.86</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>NOHA+BH₄</td>
<td>Fe(I)P</td>
<td>2.26</td>
<td>2.23</td>
<td>ND</td>
</tr>
</tbody>
</table>

Stepwise annealing of the cryo-reduced Fe(II) gsNOS sample at 145-160K for 1 min results in a decrease in the Fe(I) signal while the population of g=~4 species increases (not shown). This observation suggests that during annealing, cryo-generated Fe(I) center relaxes to thermodynamically more stable the g=~4 species which in turn disappears after annealing to temperatures above 220K.

5.3.1.3 Oxy gsNOS. The cryo-reduced oxy-heme species trapped at 77 K were shown to retain the conformations of the precursors and are sensitive EPR/ENDOR probes for studying structural features of heme centers in the diamagnetic oxy precursors. The primary cryo-generated species is peroxo Fe(III) heme intermediate (30). However, the structure of the primary cryo-reduced species trapped at 77 K depends essentially on the presence of proton donor (predominantly molecule of water) in distal side of oxy-heme moiety that is incorporated in hydrogen bonding network to coordinated O₂. In the absence of water the cryo-reduced species trapped at 77K was shown to be peroxo-Fe(III) intermediate. In this species, basic Fe(III)O₂²⁻ center adds a
proton to form hydroperoxy-Fe(III) heme intermediate as a rule at temperatures above 170 K. At this temperature, proton can migrate to peroxy ligand from a proton donor beyond the heme pocket. In contrast, in cryo-reduced oxy-heme mono-oxygenases with elaborate H-bonding network containing H₂O for delivering first proton of catalysis, protonation of peroxy ligand occurs as rule at temperatures below 77K (18, 30). Thus, in this case, the hydroperoxy Fe(III)species is trapped at 77K. In both species, the Fe(III) heme has $S = \frac{1}{2}$ and shows characteristic rhombic EPR signals with g-values within 1.91- 2.35 (30, 31). However, because of lower spin density on iron(III) in cryo-generated peroxy-Fe(III) species as compared to that in hydroperoxy-Fe(III) intermediates, the latter shows rhombic EPR signals with larger g-anisotropy (18, 31, 32).

Figure 5.5 presents EPR spectra of cryo-reduced substrate free oxy-gsNOS and its complexes with substrates and inhibitors. Cryo-reduced substrate free oxy-gsNOS shows dominant rhombic EPR signal with g-values of 2.30, 2.21 ($g_3$ is not determined because of overlapping this feature with strong radiolytically generated radical signal) (Figure 5.5, Table 5.3) characteristic of hydroperoxy ferric heme species. This observation thus indicates that protonation of the peroxy ligand in the cryo-reduced oxy-heme center occurs at temperatures below 77 K. EPR spectrum of cryo-reduced ternary oxy-NOS-Arg complex shows two EPR signals indicating the presence of two conformational sub-states in the precursor. g-Tensor components of the major signal are 2.30, 2.16 and <1.94 and are characteristic of a hydroperoxy Fe(III) heme intermediate but differ from these for the cryo-generated substrate free species (Table 5.3).
Figure 5.5: Effect of substrates and inhibitors on EPR spectra of cryo-reduced oxy-gsNOS. EPR conditions: T=2K, Modulation amplitude (Am) 2G, Microwave frequency (MF), 35GHz
Table 5.3: g-Tensor components for cryo-reduced and annealed oxy-gsNOS complexes.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>T (K)</th>
<th>Species</th>
<th>G₁</th>
<th>G₂</th>
<th>G₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxygsNOS</td>
<td>Arg</td>
<td>77</td>
<td>Major</td>
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<td>2.157</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>77</td>
<td>Minor</td>
<td>2.268</td>
<td>2.18</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>155</td>
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<td>2.25</td>
<td>1.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>155</td>
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<td>2.25</td>
<td>1.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>175</td>
<td></td>
<td>2.50</td>
<td>2.25</td>
<td>1.88</td>
</tr>
<tr>
<td>oxygsNOS</td>
<td>Arg+BH₄</td>
<td>77</td>
<td>Major</td>
<td>2.30</td>
<td>2.16</td>
<td>ND</td>
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<tr>
<td></td>
<td></td>
<td>77</td>
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<td>2.57</td>
<td>2.18</td>
<td>~1.87</td>
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<td></td>
<td></td>
<td>77</td>
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<td>2.18</td>
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<td></td>
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<td>145</td>
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<td></td>
<td></td>
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<td>2.25</td>
<td>1.88</td>
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<td></td>
<td></td>
<td>77</td>
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<td></td>
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<td>160</td>
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<td></td>
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</tr>
<tr>
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<td>Me-Arg</td>
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<td>2.26</td>
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<td></td>
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<td></td>
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<td>2.255</td>
<td>1.86</td>
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<tr>
<td>oxygsNOS</td>
<td>NO₂-Arg</td>
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<td>Major</td>
<td>2.30</td>
<td>2.20</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>77</td>
<td>Minor</td>
<td>2.27</td>
<td>2.16</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>145-175</td>
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<td>1.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>145-175</td>
<td>Minor</td>
<td>2.28</td>
<td>2.28</td>
<td>1.88</td>
</tr>
</tbody>
</table>

These distinctions in EPR properties indicate that the bound substrate perturbs geometry of oxy-heme moiety in the line with the reported absorption spectroscopy data (9, 10, 19). The minor species shows smaller spread in g-values (Table 5.3) that are characteristic of a peroxo-ferric heme intermediate. Relative contribution of this species noticeably increases in deuterated solvent (Figure 5.5), because of slowing the rate of protonation of the peroxo ligand. It is clear from data presented in Figure 5.5 and Table 5.3...
that binding of NOHA stabilizes basic peroxo ferric-heme intermediate. This infers that bound NOHA unlike Arg protects protonation of the peroxo ligand in cryo-generated species at 77 K. Interestingly, bound NOHA and Arg also have slightly different effect on g-tensor components for cryo-generated peroxo species (Table 5.3). Taken together, these observations indicate that bound Arg and NOHA differently perturb the hydrogen-bonding network to dioxygen ligand and the structure of the oxy-heme moiety. Like the EPR spectrum of cryo-educed oxy-gsNOS-Arg, cryo-reduced oxy-gsNOS-N-Me-Arg discloses hydroperoxo (major) and peroxo ferric heme species (minor) and the spectroscopic properties of the species differ noticeably from those for the respective intermediates in cryo-reduced ternary oxy-gsNOS-Arg complex (Table 5.3). Comparison of g-values for cryo-reduced oxy-NOS with bound Arg and its analogs / inhibitors presented in Table 5.3 shows that like ferric form, nature of the substituent in the Arg analog has noticeable effect on the electronic structure of the oxy-heme center. This is may be due to both direct interaction of substrate with bound dioxygen and different perturbing effect of bound arginine analogs on protein environment of oxy-heme site, including the hydrogen-bonding network to dioxygen ligand and the interaction of the proximal ligand with Fe(III).

5.3.1.4 NO complexes of gsNOS The observation of perturbing effects of bound substrates of oxy-heme moieties of gsNOS was supported by EPR studies on complexes of Fe(II)NOS with dioxygen analog NO. Figure 5.6 exhibits the EPR spectra of the NO complexes of substrate free Fe(II) gsNOS and those in the presence of Arg, N-methyl-Arg and NOHA. The spectra presented in
Figure 5.6 resemble very much those reported for respective NO complexes of mammalian n-NOS (33).

![Graph showing EPR spectra with fields in G and peaks at 2.087, 2.008, 1.97, 2.076, 2.009, and 1.983.]

Figure 5.6: Effect of Arg, NOHA and MeArg on EPR spectra of gsNOS-NO. Conditions: 30K, Am=3.2G, P=2mW, MF=9.376 GHz

NO adducts of NOS show rhombic cytochrome P450-type EPR spectra typical of a hexa-coordinate NO-heme complex with non-nitrogenous proximal axial heme ligand (33). Binding of Arg and its analogs affect rhombicity of g-tensor and resolution of $^{14}$N hyperfine structure for bound NO in the EPR spectra (Figure 5.6). More significant changes are seen in the EPR spectra of ferrous-NO complexes in the presence of NOHA and N-methyl-Arg. The spectra are
less anisotropic than those of the substrate free and the L-Arg bound NO complexes, with distinct changes in g-values. Previous studies on ferrous heme-NO complexes have shown that their EPR properties are sensitive to the Fe-NO geometry (33, 34). The reported structural data for different complexes of mNOSs and bNOSs with diatomic ligands (NO, CO, O2) show that bound substrates are in close proximity to the diatomic ligands suggesting that the substrates can perturb the coordinated NO (35, 36). These data thus support our observations on effect of substrates on oxy-heme site in gsNOS and reveal structural similarities between heme sites in gsNOS and mNOSs.

5.3.2 Annealing of the cryo-reduced oxy gsNOS intermediates

Cryo-reduced samples were progressively annealed at different temperatures as described (18) and examined by EPR after each step. As shown previously these experiments enable one to trap and characterize intermediates that occur along the reaction pathway of NOS mono-oxygenase cycle (17, 18, 37).

5.3.2.1 Substrate free oxy-gsNOS. The simplest results were obtained with substrate free oxy-gsNOS. Figure 5.7 shows EPR spectra of cryo-reduced oxy-gsNOS during progressive step annealing at increasing temperatures. During annealing, EPR signal of cryo-generated hydroperoxy ferric NOS intermediate decreases with simultaneous increase of a new EPR signal with g-values of 2.42, 2.28 and 1.91 characteristic of low spin aqua ferric gsNOS (Figure 5.7).
Figure 5.7: X-band EPR spectra of cryoreduced oxyNOS annealed at indicated temperatures. Instrument: 28K, Am=10G, 10mW, 9.375 GHz

The relaxation of cryo-generated hydroperoxy intermediate into resting state is completed at 175 K and this process proceeds without formation of other spectroscopically detectable intermediates as it happens in case of cryoreduced ternary oxy-gsNOS-substrate complexes.

5.3.2.2 Ternary oxy-gsNOS-Arg complex. Figure 5.8 shows EPR spectra taken during annealing of the cryo-reduced ternary oxy-gsNOS-Arg complex at successively higher temperatures for different periods of time. During this
process, the primary hydroperoxo-ferric species almost completely decays at relatively low temperature of 145 K.

Figure 5.8: Fig.5 X-band EPR spectra of cryoreduced oxyNOS+Arg annealed at indicated temperatures for 1 min. Conditions: T=20K, A=10G, P=2 mW, 9.365 GHz

The decay of the hydroperoxo-ferric species during this annealing is accompanied by parallel increase of two new low-spin rhombic signals with close g-tensor components: dominant [2.50, 2.25, 1.88] and minor [2.48, 2.25, 1.91] (referred to as 2.5 and 2.48 species, respectively) (Figure 5.8, Table 5.3). Further annealing at 175 K results in conversion 2.48 species into 2.5 species (Figure 5.6) and the intensity of EPR signal of the latter does not change after
annealing at 200 K for 1 min (Figure 5.6). At 230 K the 2.5 species disappears and EPR spectrum of the resting ferric state appears (Figure 5.6, Table 5.3). HPLC analysis of OPA derivatized standards Arg, NOHA and Cit showed expected elution profiles for the respective molecules (Figure 5.9).

![HPLC profile of standards](image)

Figure 5.9: HPLC analysis shows expected elution times of OPA derivatized standard molecules.

Similar analysis of the irradiated oxy-gsNOS-Arg sample annealed at room temperature showed that the sample contained ~0.3 mM NOHA and ~0.6 mM Arg (Figure 5.10). In this experiment the amount of NOHA formed is close to that of the cryo-generated hydroperoxo-ferric species which is ~0.4 mM (at a dose of 3Mrad) inferring close to quantitative conversion of bound Arg into NOHA by the cryo-generated hydroperoxo-ferric-NOS species.

![HPLC profile of irradiated sample](image)

Figure 5.10: HPLC of irradiated and annealed oxy-gsNOS-Arg sample reveals that product NOHA was formed in the reaction.

The EPR properties of the g 2.48 and 2.5 intermediates resemble these for the intermediate arising during annealing cryo-reduced oxy-eNOS-Arg complex.
assigned to adduct of low-spin ferric NOS with formed NOHA as well as for the complex of ferric P450cam with the product 5-hydroxycamphor formed during annealing cryo-reduced ternary oxy-P450-camphor complex. One can propose that during conversion the primary hydroperoxy species into the g 2.5 intermediate, NOHA coordinated to ferric heme is formed. On the other hand similar spectra show the low spin form of citrulline bound ferric NOS. In addition, alkaline forms of thiolate ligated ferric P450cam and CPO were also reported to exhibit rhombic low-spin EPR signals with similar g-values (24, 38). However, annealing pattern for the g 2.5 species differs noticeably from that for known cryo-generated hydroxy Fe(III)-heme intermediates (38). As a rule, protonation of hydroxy heme iron(III) centers in studied hemoproteins including CPO occurs at temperatures below 170 K while the g 2.5 species disappears at temperatures above 200 K. To better understand the mechanism of this process and particularly the nature of the g 2.5 intermediate we studied solvent KIE for decay of the primary species and ENDOR spectra of the intermediates emerging during annealing.

Figure 5.11: Kinetics of decays g 2.3 species at 145 K: oxyNOS+Arg pH 8.5 red; oxyNOS+Arg in D2O pH 8.1 green; oxyNOS+Arg pH 7.5 -dark red and oxy NOS+Arg +BH4 pH 8.5 - blue.
As seen in Figure 5.11, the half-life of the decay of the cryo-generated hydroperoxo-ferric NOS-substrate complex at 145 K increases by about 8 times in deuterated solvent. This significant solvent KIE suggests that under studied conditions the limiting step in conversion of the primary hydroperoxy ferric intermediate into the g 2.5 species is proton transfer as it is expected for proton assisted conversion of hydroperoxy intermediate into compound I, which then rapidly reacts with protein bound Arg to form NOHA that remains coordinated to ferric heme at low temperatures.

This supposition is supported by $^1\text{H}$ and $^{15}\text{N}$ ENDOR studies. Figure 5.12 and Figure 5.13 show 2D field-frequency set of $^1\text{H}$ ENDOR spectra of cryo-reduced ternary oxy NOS-Arg complex and the g 2.5 species collected at selected g-values across their EPR envelopes.

![Figure 5.12: H ENDOR spectra of cryoreduced oxyNOS + Arg taken at indicated g-values. ENDOR-spectra of cryoreduced oxyNOS+Arg in $^2\text{H}_2\text{O}$ are shown by dotted line. Conditions: T=2K, 35 GHz, Am=1G, rf sweep rate of 0.5MHz/sec, RF power 5W, 15scans.](image-url)
Detailed analysis of ENDOR spectra for the different intermediates arising during mono-oxygenase cycles of cryo-reduced oxy-P450cam and oxy-eNOS-substrate complexes were done previously therefore here, many spectroscopic details not related directly with interpretation of the results are not mentioned. The cryo-reduced intermediate trapped at 77 K shows strongly coupled exchangeable in $^2$H$_2$O proton ENDOR signals with maximum hyperfine coupling $A = 10$ MHz characteristic of the hydroperoxo proton (18).

![ENDOR spectra](image)

Figure 5.13: H ENDOR spectra of cryo-reduced oxyNOS+Arg after annealing at 175K for 1 min (g 2.5 species) taken at indicated g-values. Conditions: T=2K, 35 GHz, Am=1G, rf sweep rate of 0.5MHz/sec, RF power 5W, 15scans.

The g; 2.5 species shows quite distinct H ENDOR pattern presented in Figure 5.13. This species discloses exchangeable H ENDOR signals with significantly smaller maximum hyperfine coupling of Amax = 7.5 MHz. This magnitude of
$A_{\text{max}}$ is essentially less than 10-12 MHz, characteristic of protons from water or hydroxide ligands coordinated to low spin Fe(III) heme of NOS (Figure 5.3). The strongly coupled proton signal in the ENDOR pattern for the $g = 2.5$ species is also less, at 9.2 MHz reported for hydroxyl of 5-hydroxy camphor coordinated to ferric P450cam (18). Taking into account that $pK_a$ for hydroxy camphor and hydroxy Arg (NOHA) are $\sim 12$ (18) and 8.1 (8) the H ENDOR signal thus can be assigned to the NOHA hydroxyl with broken or weakened O-H bond coordinated to heme iron (III) as proposed by Mansuy and coworkers (39). To confirm this assignment we have applied $^{15}$N Q-band Mims pulsed ENDOR spectroscopy to the intermediates formed during annealing cryo-reduced oxy-gsNOS with Arg isotopically labeled with $^{15}$N at the terminal guanidino nitrogen atoms. As shown previously these measurements enable to estimate the distance between $^{15}$N-labeled substrate bound to the enzyme and iron(III) (40).

Figure 5.14 presents Mims pulsed $^{15}$N spectra for the $g = 2.5$ species formed during annealing the cryo-reduced ternary complex of oxy-gsNOS with $^{15}$N Arg and with natural abundance ($^{14}$N) Arg, for comparison. Mims pulsed ENDOR spectra of the species with $^{15}$N labeled substrate show very well resolved doublets centered at $^{15}$N larmor frequency. These signals are absent for sample that contains natural abundance of Arg confirming that they arise from $^{15}$N labeled substrate. The maximum hyperfine interaction of $A_{\text{max}}=0.7$MHz is observed at $g_1$ and can be used for an approximate estimation of the minimum Fe-$^{15}$N distance. Analysis of the data gives a distance of $\sim 3.5$ Å, which is comparable with that for NOHA hydroxyl coordinated to heme iron. It is worth noting that the cryo-generated ternary hydroperoxo ferric
NOS-[\textsuperscript{15}N]-Arg shows no detectable \textsuperscript{15}N ENDOR signals. The latter might be due to longer distance between heme iron and guanidine N of Arg separated by the peroxide ligand.

\[ \begin{array}{c}
\text{\textsuperscript{15}N} \\
\text{\textsuperscript{14}N} \\
10900 \text{ G} \\
12000 \text{ G} \\
13000 \text{ G} \\
\end{array} \]

\[ v - v (\textsuperscript{15}N), \text{MHz} \]

Figure 5.14: \textsuperscript{15}N Q-band Mims pulsed ENDOR spectra for the the g 2.5 species forming during annealing cryoreduced oxyNOS+\textsuperscript{15}N-Arg (solid line) and oxyNOS+\textsuperscript{14}N-Arg (dotted line) taken at indicated magnetic fields. T=2K.

Collectively, the presented H and N ENDOR data indicate that in the g 2.5 species heme iron is coordinated by a product formed during the relaxation of the cryo-generated hydroperoxo-ferric NOS–Arg intermediate. Identification of the of the g 2.5 species structure is very important for elucidation of active oxy intermediates in the mechanism of Arg oxidation.
5.3.2.3 Effect of BH₄. Binding BH₄ exerts a little influence on g-tensor components of the cryo-generated hydroperoxy ferric-NOS-Arg complex but causes appearance a new small more rhombic EPR signal with g-values of 2.57, 2.18 and ~1.87 which completely disappears after annealing at 145 K for 3 min (Figure 5.15, Table 5.3).

![EPR spectra](image)

**Figure 5.15:** X-band EPR spectra of cryo-reduced oxyNOS-Arg-BH₄ annealed at indicated temperatures. Instrument: 28K, Am=10G, 10mW, 9.375 GHz

The cryo-generated hydroperoxy intermediate decays several times faster than that in the absence of BH₄ at 145 K (Figure 5.11). This process is accompanied by appearance and growth of two well-resolved EPR signals from low-spin ferric-heme form with g-tensors $g=[2.46, 2.246, 1.907]$ and $g=[2.50, 2.25, 1.88]$; these are denoted as the $g_1$-2.46 and $g_1$-2.50 (Figure 5.15, Table 5.3). In
addition, in the beginning of annealing, small EPR signals with g-values of 2.36, 2.18 and ~1.926 and 2.30, 2.21 and ~1.93 also appear (the g₁, 2.36 and 2.30 species, respectively) that then decay during this process (Figure 5.15). At the early stage of annealing the g₁-2.46 is dominant but this species converts into the g₁-2.5 intermediate upon annealing at 195 K (Figure 5.15). Further stepwise annealing to 230 K causes the loss of the g-2.5 species and formation of the resting ferric state (not shown). Spectroscopic properties of these species are very similar to those for the g 2.5 species formed during relaxation of the BH₄ free hydroperoxy Fe(III)NOS-Arg intermediate and they can be attributed to two different conformational sub-states of the ferric-NOS-product intermediates like that as it is observed during annealing of the cryo-reduced ternary complexes of oxy-eNOS-Arg (17) and oxy D251N P450-camphor (18).

5.3.2.4 *Ternary oxy-gsNOS-Arg analogs / inhibitors.* Annealing pattern for the cryo-reduced oxy-gsNOS with NO₂-Arg resembles very much that for the cryo-generated substrate free hydroperoxy ferric gsNOS (Figure 5.16). Like substrate free hydroperoxy-gsNOS, the initial species observed at 77 K reduction of the oxy-gsNOS inhibitor complex converts to low-spin aqua ferric state upon annealing to 175-240 K and this process occurs without formation other EPR detectable intermediates (Figure 5.16). This observation indicates that this Arg analog inhibitor probably does not interact with active oxy species arising during relaxation of cryo-generated hydroperoxy Fe(III)NOS intermediate or the active oxy species does not form in the presence of the inhibitor.
Figure 5.16: EPR spectra of the cryo-reduced ternary oxy gsNOS+NO₂Arg complex annealed at indicated temperatures. Instrument: 28K, Am=10G, 10mW, 9.375 GHz

In contrast, during annealing the cryo-reduced oxy-NOS generated in the presence of the other inhibitor, N-methyl-Arg, formation of new low-spin ferric heme species are observed. Like the Arg bound intermediate, progressive annealing of the cryo-reduced oxy-gsNOS-methyl-Arg at 145 K leads to decay of the the primary g, 2.34 species (Figure 5.17) and this process is accompanied by appearance of a new EPR signal from low-spin ferric species with g-tensor, g=[2.26, 2.19,~1.94] (Figure 5.17, Table 5.3).
Figure 5.17: X-band EPR spectra of cryo-reduced oxyNOS+Me-Arg annealed under indicated conditions. Instrument: 28K, Am=10G, 10mW, 9.375 GHz.

Further step-wise annealing to 185 K causes the loss of the $g_{ii}$ 2.26 species and an appearance of several new EPR signals with close g-tensor components, reminiscent of the $g_{ii}$ 2.5 species formed during annealing cryo-generated hydroperoxo-ferric NOS-Arg intermediate (Figure 5.17, Table 5.3). This species converts into resting ferric state upon annealing at 225 K (not shown). HPLC of the annealed sample reveals a strong N-methyl-Arg peak and a new small peak (not shown) that might be assigned to the product. The EPR properties of
the $g_i$ 2.26 species resemble those for the peroxo ferric heme species. However, it is hard for us to imagine how hydroperoxo ferric heme intermediate converts into peroxo species. This is possible only in the presence of very strong base near the active site which will compete with one-electron reduced oxy-heme for proton. In that case, it remains unclear why in the primary product of cryo-reduction, the peroxo ligand of the heme iron (III) is protonated. The other explanation is based on the notion that this species is ferric NOS-product complex having non-equilibrium conformation. At higher temperatures of 165 K this complex relaxes to more equilibrium conformational state showing the $g_i$ 2.5 signal.

![Graph](image)

Figure 5.18: Time courses of decays of the cryoreduced ternary oxy gsNOS+MeArg complex in 50% EG/H$_2$O pH 8.5 (A) and 50% d$_2$-EG/ D$_2$O pH 8.1 (B) at 145K.

Mansuy and coworkers reported that some N-hydroxyguanidines form low-spin complexes with ferric NOS which display rhombic EPR signal with $g$-values of 2.35-2.38, 2.25 and 1.93 (39). These notions are supported by solvent KIE and H ENDOR studies. The decay of the cryo-generated hydroperoxo-
ferric NOS-Me-Arg slows down in deuterated solvent by 6-8 fold (Figure 5.18). This solvent KIE is comparable with that for Arg bound NOS. In contrast, solvent KIE for conversion the $g_1$ 2.26 species into the $g_1$ 2.5 intermediate at 165K is less than 2. The difference in the solvent KIE for the compared processes infers that like in the Arg reaction, formation of the $g_1$ 2.26 species is associated with hydroxylation of bound N-Me-Arg.

Figure 5.19: H ENDOR spectra taken at different magnetic fields for cryoreduced oxyNOS+MeArg and after its annealing at 156K for 3 min (g 2.26 species) and at 170K for 1 min (g 2.5 species). Conditions: T=2K

$^1$H ENDOR spectra of cryo-reduced oxy-gsNOS-N-Me-Arg show well resolved strongly coupled exchangeable $^1$H ENDOR signal from hydroperoxy
ligand coordinated to heme Fe(III) with maximum hyperfine coupling of 11 MHz (Figure 5.19). In contrast, the $g_i$ 2.26 species and particularly the $g_i$ 2.5 species show less resolved exchangeable H ENDOR signals with $A_{\text{max}}$ less than 8 MHz (Figure 5.19) i.e. the spectroscopic properties of the species are close to those for the low-spin complex of NOS with NOHA forming during relaxation of cryo-reduced oxy-gsNOS-Arg.

Figure 5.20: EPR spectra of cryoreduced oxyNOS+NOHA annealed at indicated temperatures. Instrument: 28K, $A_{m}=10 G$, 10mW, 9.375 GHz

5.3.2.5 OxyNOS+NOHA. Unlike the oxy-gsNOS complexes of substrate / analogs considered above, the 77 K reduction of oxy-gsNOS-NOHA complex
produces a peroxo ferric species with g-values (Table 5.3) very close to those for reported cryo-reduced oxy-eNOS-NOHA (17). This infers that perturbations in hydrogen bonding network by bound NOHA impedes delivery of a proton to the basic peroxo ligand of ferric heme at 77 K. Progressive annealing at to 160 K causes peroxo-gsNOS-NOHA to disappear. At the earlier stage of the process, only a new small signal with g-values of 2.36, 2.21 and 1.93 appears (Figure 5.20, Table 5.3). As followed from data presented in Figure 5.20 and Figure 5.21, this signal decays upon further annealing at 160 K.

Figure 5.21: The time courses of the changes of the 2.36 species during annealing the cryo-reduced oxy-gsNOS-NOHA in 50% EG/ H₂O pH 8.5 (red), 50% d₂ EG/D₂O pH 8.1 (green) and in the presence of 2mM BH4 in 50% EG/ H₂O pH 8.5 (black).

In approximately 5 min after beginning the process, one can see the appearance of other two new rhombic EPR signals with close g-tensors of \(g=[2.467, 2.26, 1.905]\) and \(g=[2.487, 2.26, 1.905]\) (denoted as \(g_1\), 2.47 species) that
continue to increase until complete decay of the primary peroxo ferric heme intermediate (Figure 5.20, Figure 5.22).

![Graph showing kinetics of decay](image)

Figure 5.22: Kinetics of decay of the cryoreduced oxy gsNOS+NOHA (A) and oxy gsNOS+NOHA+BH4 (B) and the formation of the 2.47 species (C and D, respectively) during annealing at 160K.

The g₁ 2.47 species disappears with measured rate at temperatures above 170 K and this decay is accompanied by appearance of a new narrow EPR signal with g 2.079-1.969 (Figure 5.20, Figure 5.23) which is very similar to that for substrate free Fe(II)NOS-NO complex presented in Figure 5.6.

![EPR spectrum](image)

Figure 5.23: EPR spectrum of ferrous gsNOS-NO complex arising during annealing the cryo-reduced oxy-gsNOS-NOHA complex at 180K for 20min.
We have found that decay of the g 2.47 species and formation NOS-NO species are described by close kinetics (Figure 5.24) suggesting that during annealing the g 2.47 species converts directly into the complex.

Figure 5.24: Kinetics of decay of the 2.47 species (50% EG/H$_2$O pH 8.5 (blue) 50% d$_2$ EG/D$_2$O pH 8.1 (red)) and the formation of Fe(II)NOS-NO complex (50% EG/H$_2$O pH 8.5 brown) during annealing the cryoreduced oxyNOS+NOHA complex at 180K.

HPLC analysis of the cryo-reduced oxy-gsNOS-NOHA sample annealed to 273 K shows that the cryo-generated peroxo NOS intermediate quantitatively converts bound NOHA into Cit (Figure 5.25).

Figure 5.25: HPLC analysis of irradiated and annealed oxy-gsNOS-NOHA sample shows quantitative conversion of NOHA to Cit.

As seen from data presented in Figure 5.26, bound BH$_4^-$ has no noticeable effect on the EPR properties of the cryo-reduced ternary oxy-gsNOS-NOHA
complex but affects the annealing pattern appreciably (Figure 5.26, Table 5.3). Bound BH$_4$ noticeably slows down decay of the peroxo-ferric NOS intermediate and increases lag period in kinetic of the $g_t$ 2.47 species formation (Figure 5.22). In addition during annealing to 160 K, along with the $g_t$ 2.47 species the appearance of low spin signal with g-value of 2.42, 2.28 and 1.91, which can be attributed to aqua ferric heme NOS is observed (Figure 5.26, Table 5.3).

Figure 5.26: EPR spectra of cryoreduced oxyNOS+NOHA+BH4 annealed at indicated temperatures. Instrument: 28K, Am=10G, 10mW, 9.375 GHz

Figure 5.27 presents data of kinetic measurements for decay the cryo-generated peroxo ferric-NOS-NOHA intermediate in deuterated and non-
deuterated solvents and at different pH. In deuterated solvent this decay slows down by factor of ~4-5. The solvent KIE magnitude suggests that delivery of proton to peroxo heme moiety should be limiting step in this process at 160 K. In this context it is surprisingly that change of pH from 8.5 to 7.5 has a little effect on the kinetics.

![Graph showing decay kinetics with pH and D₂O](image)

**Figure 5.27: Effect of pH and D₂O on kinetics of decay of the cryogenerated peroxo-ferric NOS+NOHA intermediate at 160K. 50%EG/H₂O buffer pH 8.5(red) and pH 7.5(brown); 50% d₂EG/D₂O buffer pH 8.1 (blue)**

Orientation-selective 2D field / frequency ENDOR pattern for the cryogenerated NOHA bound peroxo-ferric NOS intermediate discloses strongly coupled exchangeable proton ENDOR signal with maximum hyperfine coupling $A_{max}$ at $g_2$ of ~9.5 MHz, which can be attributed to hydrogen atom of possibly NOHA hydroxyl forming H-bond with the peroxo ligand (Figure 5.28). Note the $A_{max}$ magnitude is similar to that for the cryo-generated peroxo ferric myoglobin and adult hemoglobin α-chains although less than 15-19 MHz observed for instance for the cryo-generated peroxo ferric hemoglobin β-chains (30). The difference might be due to different H-bond distance that in
turn affects both the spin density on the peroxo ligand and spin transfer to hydrogen bonded proton donor.

Figure 5.28: H ENDOR spectra for the cryo-reduced oxy-gsNOS-NOHA complex in H₂O (solid line) and D₂O (dotted line) and for the g 2.47 species arising during annealing the primary species at 160K for 350 min. Conditions: T=2K

In contrast to the g₁ 2.5 species trapped during annealing cryo-reduced oxy-gsNOS in presence of Arg and N-Me-Arg the g₁ 2.47 species shows well resolved strongly coupled H ENDOR signal with Amax ~ 10 MHz. (Figure
5.28) which is comparable with that for low spin aqua ferric NOS. Some
differences between $A_{\text{max}}$ for compared species may be due to perturbing
effect of Cit that is being formed.

5.4 DISCUSSION AND CONCLUSIONS

5.4.1 Conversion of Arg to NOHA The two-step reaction that converts Arg to
NO and Cit is reasonably complex and several mechanisms have been
suggested. The first step has long been suspected to involve a ferryl-oxo
intermediate. We have shown direct evidence that this intermediate is the
heme-oxyl species that is responsible for oxidation of Arg to NOHA. Our
observations of the temperature annealing reaction of cryo-reduced oxy-
gsNOS-Arg indicates the mechanism shown in Figure 5.29. Fe(III) gsNOS (A)
is converted to Fe(II) gsNOS (B) by reducing agents (dithionite). On bubbling
oxygen gas, the Fe(II)-O$_2$ complex (C) forms, which in the presence of
substrate probably assumes in the Fe(III)-O$_2$' (Ferric-superoxy) form (D). On
further reduction (in this case, radiolytic cryo-reduction), the heme-peroxy
complex (E) forms, which in the presence of Arg, forms a heme-hydroperoxy
intermediate (F). The rate of decay of this species shows a KIE of 8 when the
reaction is performed in deuterated solvent and a decrease in pH from 8.5 to
7.5 also accelerates of the decay of this species. This suggests further
protonation of the hydroperoxy species as the rate limiting step at 145 K. Loss
of water follows giving rise to the highly unstable reactive intermediate, the
ferryl-oxo complex (G). This intermediate then inserts an oxygen atom into the
N-H bond, and generates a product species (H) in a non-equilibrium geometry
(product NOHA in complex with Fe(III) heme).
Figure 5.29: The proposed mechanism for oxidation of Arg to NOHA. Details in text.

Formation of H was suggested by similarity to previously observed g values for a similar complex of hydroxy-camphor with Fe(III)-cyp450cam. This was further corroborated by $^{15}$N mims pulsed ENDOR spectroscopy on the same complex in a reaction that utilized $^{15}$N labeled Arg. A coupling of 0.7 MHz is observed between the heme center and substrate, which suggests that the Fe-$^{15}$N distance is $\sim$3.5 Å. $^1$H ENDOR of this species showed strong proton coupling similar to that observed for hydroxy-camphor-Fe(III)-cyp450cam. Hence we characterized this complex as NOHA hydroxyl complexed with
Fe(III)-heme. Formation of such a complex is only possible after formation of ferryl-oxo type intermediate, as NOHA will not coordinate the heme iron under equilibrium conditions. This is further evidence that the conversion of Arg to NOHA involves ferryl-oxo intermediate. At temperatures above 230 K, H relaxes to NOHA bound in the active center with the hydroxyl no longer complexed with Fe(III) heme. At the end of cryo-annealing, we are left with Fe(III) heme. The formation of product NOHA in the cryo-reduced and annealed samples was further confirmed by HPLC analysis of OPA derivatized amino acids from the sample.

5.4.2 Conversion of NOHA to Cit In the second step of the NOS reaction cycle, NOHA is further oxidized to cit and NO (Figure 5.30). The initial species A through E are identical to the Arg reaction. However, after cryo-reduction, the heme-oxo species observed in the presence of NOHA is the Fe(III)-heme-peroxy (E) species and not the Fe(III)-hydroperoxy species. This suggests that the substrate NOHA prevents the protonation of the Fe(III)-heme-peroxo species and this dictates the reaction that follows. The decay of E initially results in a small signal with $g_z = 2.36$, which then forms two new strong close signals with $g_z = 2.47$. A 1:1 conversion kinetics of the Fe(III)-peroxo complex to this 2.47 species is observed (Figure 5.27). This 2.47 species shows substantial $^1$H coupling of $\sim 10$ MHz, which is comparable to that of aqua-Fe(III)-gsNOS. A Fe(III)-H$_2$O complex has a EPR signal with $g_z = 2.42$ (Figure 5.3, Figure 5.7). If we consider the mechanism proposed in Figure 5.30, a Fe(III)-heme coordinated with a hydroxyl group, that is either hydrogen bonded to HNO or Cit could have the observed $g_z$ value of 2.47. This complex also converts in a 1:1 fashion (Figure 5.24) (shows no KIE in a deuterated
solvent) to a new intermediate that can be characterized as the Fe(II)NOS-NO complex (Figure 5.6, Figure 5.20, Figure 5.23).

Figure 5.30: The proposed mechanism for conversion of NOHA to Cit and NO. See text for details.

The nature of the 2.36 species, the formation and decay of which depends on D/H exchange and modestly on the protons from solvent, is unclear, but
could be G or H. If we consider that the hydrogen in the hydroxyl group of NOHA is exchanged to a deuterium when added to a protein in a deuterated solvent, the rate-limiting step in G → I may be the transfer of the proton / deuterium from O to N during formation of HNO and hydroxyl-Fe(III)-gsNOS.

However, we cannot rule out the involvement of a ferryl-oxo intermediate in the conversion of NOHA to Cit, without characterization of the $g_z = 2.36$ species. It is possible that the 2.47 species is the carbonyl group of the product Cit coordinated to Fe(III) NOS, that could be a result of the oxidation of NOHA by a ferryl-oxo complex. The strongly coupled proton from $^1$H ENDOR measurements of this 2.47 species may derive from either HNO or a proton or a water molecule hydrogen bonded to the Cit carbonyl group. This could be checked by using $^{13}$C-NOHA, which would give an ENDOR signal if the carbonyl group is coordinated to Fe(III)-NOS. Whether the 2.47 species is a hydroxyl group or the carbonyl of Cit, this species (I) is partially displaced by NO$^-$ (Figure 5.24), resulting in a Fe(II)-NOS-NO complex (J). Alternatively, the HNO diffuses away and the hydroxyl group becomes protonated generating Fe(III)-NOS coordinated to a water molecule. The final EPR spectrum during annealing of cryo-reduced oxy-gsNOS-NOHA complex (Figure 5.20) suggests that both these products are formed ($g_z$ values 2.42 for aqua-Fe(III)-NOS (K), and 2.079 for Fe(II)-NOS-NO complex (J)).
REFERENCES


CHAPTER 6

THE STRUCTURE OF YqeH: AN AtNOS1/AtNOA1 ORTHOLOG THAT COUPLES GTP HYDROLYSIS TO MOLECULAR RECOGNITION

6.1 ABSTRACT

AtNOS1/AtNOA1 was identified as a nitric oxide generating enzyme in plants, but that function has recently been questioned. To resolve issues surrounding AtNOA1 activity, we report the biochemical properties and a 2.36 Å resolution crystal structure of a bacterial AtNOA1 ortholog (YqeH). Geobacillus YqeH fused to a putative AtNOA1 leader peptide complements growth and morphological defects of Atnoa1 mutant plants. YqeH does not synthesize nitric oxide from L-Arginine, but rather hydrolyzes GTP. The YqeH structure reveals a circularly permuted GTPase domain and an unusual C-terminal β-domain. A small N-terminal domain, disordered in the structure, binds zinc. Structural homology among the C-terminal domain, the RNA-binding regulator TRAP, and the hypoxia factor pVHL define a recognition module for peptides and nucleic acids. TRAP residues important for RNA binding are conserved by the YqeH C-terminal domain, whose positioning is coupled to GTP hydrolysis. YqeH and AtNOA1 likely act as G-proteins that regulate nucleic acid recognition and not as nitric oxide synthases.

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6.2 INTRODUCTION

_Arabidopsis thaliana_ Nitric Oxide Synthase 1 (AtNOS1) was originally identified as a plant enzyme capable of producing nitric oxide (NO) from the amino acid L-arginine (Arg) (1). In plants, NO functions in many processes including seed germination, hormone responses, respiration, root development, leaf expansion, fruit maturation, senescence, abiotic stress response, cell death and disease resistance (2-5). In particular, the role of NO in plant-pathogen interactions has received considerable attention (6-8). Plant extracts generate NO and citrulline in an Arg-dependent manner and this activity can be blocked by animal NOS inhibitors (9-14). Arg dependent NO synthesis is important because the well-studied animal nitric oxide synthases (NOSs) produce NO from this substrate in an active center that contains heme and tetrahydrobiopterin (15, 16). NOS inhibitors prevent some NO-mediated responses, such as ABA-induced stomatal closure (1) and also compromise the resistance to pathogens (17).

AtNOS1 was the second of two plant enzymes reported to catalyze the conversion of Arg to NO (1, 18), but in both cases the results have not been reproduced (8, 19-21). AtNOS1 was identified from _A. thaliana_ based on homology to a hypothetical snail NOS that appeared to synthesize NO (22). Despite virtually no sequence similarity with animal NOSs, mutation of the _AtNOS1_ gene generates a growth phenotype that can be rescued by NO donor compounds. In addition, chemical probes sensitive to NO show reduced activation in the knock-out mutant (1). Genetic studies (23) further demonstrate that the Atnos1 mutant is more susceptible to the pathogen _Pseudomonas syringae_ than wild-type plants. However, consistent with our own results, several groups have stated that they cannot reproduce NO
synthase activity with host-derived or recombinant AtNOS1, calling into question the true function of this protein and resulting in its redefinition as AtNOA1 (for Nitric Oxide Associated Protein) (8, 21).

AtNOA1 is a 561 residue protein that contains four sequence motifs characteristic of GTP-binding proteins, such as P21-Ras, Rho, Rac, Cdc42, and G domains (24-26). However, the order of motifs in the protein sequence indicates an unusual circular permutation of the polypeptide found only in a small subclass of GTPases of poorly understood function (24). Proteins containing circularly permuted G-domains (CPGs) are prevalent in bacteria (YlfE, YqeH, YjeQ, YawG, MJ1464), but can also be found in yeast, plants and even humans (e.g. LSG1) (25). CPGs also belong to the HAS-GTPase subfamily, which have a Hydrophobic Amino acid Substitution (HAS) in the place of a key hydrophilic residue that participates directly in GTP hydrolysis (often a Gln or His residue). Whereas AtNOA1 conserves the central HAS GTPase region, the protein also contains additional N- and C-terminal domains, neither of which have any relationship to other members of this family, except the shorter YqeH (~360 aa). In fact, YqeH is likely to have a very similar domain structure as AtNOA1 (22.8% identity, 33.6% similarity, for the Geobacillus stearothermophilus YqeH (GsYqeH). Both proteins contain an N-terminal region that harbors four cysteines, (CX₂CX₂₅₋₃₃CX₂C) and secondary structure motifs consistent with the Treble-clef family of zinc-binding proteins (24). The conserved C-terminal regions of both AtNOA1 and YqeH have no detectable homology to any domain of known structure. All of these features are conserved in the rice (Oryza sativa), and tomato (Solanum lycopersicum) homologs of AtNOA1 (63.1% and 59.9% amino acid identity, respectively).
The similarity between AtNOA1 and YqeH is even more striking if a putative N-terminal mitochondrial targeting motif (27) is excluded from the comparison (29% identity, 43% similarity). The unusual arrangement of GTPase signature motifs and the unusual pendant domains make AtNOA1/YqeH interesting not only in the context of NO signaling in plants but also for understanding G-protein structure and function.

The function of YqeH in bacteria is not well understood. Some members of the CPG family (i.e. YlqF) have been implicated in ribosome biogenesis (28-30), but they contain C-terminal RNA-binding domains not found in YqeH/AtNOA1. Both YlqF and YqeH are essential for cell growth in Bacillus subtilis. They have been shown to bind [32P]GTP (31), although GTPase activity was not directly demonstrated for either of the two proteins. The B. subtilis yqeH mutant is lethal, while reduced YqeH expression increases chromosomal replication (31). YqeH participates in the biogenesis of the 30S ribosome subunit (32) and assists in 50S ribosome assembly (33). A high-throughput screen in yeast found that a distant homolog of YqeH interacts with a ribosomal protein (31, 34, 35). There is currently little data available about AtNOA1/YqeH mammalian homologs, aside from some localization studies that place them in the mitochondria (27). Nevertheless, it is clear that these proteins form a unique family that is broadly represented in biology. Their role in NO metabolism may very well extend to additional organisms.

Factors involved in bacterial ribosome assembly are potential targets for new anti-bacterial drugs (36-39). The fact that YqeH is found in gram-positive bacterial pathogens, coupled with the lethality caused by its deletion
in *B. subtilis*, make it attractive as a target for design of inhibitor drugs. Molecular structures of YqeH with bound substrates and inhibitors could aid such efforts.

It has been previously indicated that AtNOA1 does not synthesize NO (8, 21), but no experimental data was published along with these reports. Furthermore, the putative GTPase activity of the enzyme has not been characterized, nor have its other biochemical properties. In an effort to address the issues surrounding the function of ANOA1, we report the 2.36 Å resolution crystal structure of the YqeH homolog from *G. stearothermophilus* and further investigate its biochemistry. We also show that these data are highly relevant to AtNOA1 because bacterial YqeH rescues growth and morphological defects of *Atnoa1* mutant plants. The combined results indicate that YqeH/AtNOA1 is very unlikely to have NO synthase activity, but rather appears to be a unique regulator capable of coupling GTP hydrolysis to nucleic acid and/or protein recognition.

6.3 EXPERIMENTAL PROCEDURES

6.3.1 Materials Sodium citrate and sodium chloride were obtained from Mallinckrodt, polyethylene glycol 5000 mono methyl ether (PEG5K MME) from Fluka, Tris (hydroxymethyl) aminomethane (TRIS) from Fisher Scientific and all other chemicals were obtained from Sigma-Aldrich, unless otherwise noted.

6.3.2 Cloning and Expression of GsYqeH The YqeH gene of *G. stearothermophilus*
(ATCC strain number 12980) was amplified from genomic DNA by PCR (with Phusion polymerase from New England Biolabs) and cloned into the pET28 (Novagen) expression vector between NdeI and XhoI. GsYqeH was then expressed in E. coli BL21(DE3) cells with an N-terminal 6-His tag. Proteins were purified using Ni-chelate chromatography and then size-exclusion chromatography (Superdex 75) after removal of the 6-His tag with thrombin. GsYqeH was concentrated to ~12 mg/ml in 50 mM TRIS pH 7.5, 150 mM NaCl as estimated by the Bradford Assay.

Selenomethionyl protein was overexpressed in E. coli B834(DE3) cells, which are auxotrophic for methionine (40). An overnight culture in LB was spun down, washed twice with autoclaved water, and then added to the Se-Met growth medium (M9 minimal media supplemented with 19 standard amino acids and L-selenomethionine at 50 mg/L), which was then incubated at 37 °C for 6 hours after induction with IPTG before harvesting cells. The protein was purified as above. DTT (10 mM) was present in all buffers.

The Thr-Ala and Cys-Ser substitutions in GsYqeH were produced using the QuickChange Mutagenesis Kit from Stratagene.

6.3.3 GTPase Assays The GTPase reactions were performed with 10 µM protein in a buffer that contained 50 mM TRIS pH 7.5, 150 mM NaCl, 1 mM MgCl₂, and 1 mM GTP at 37 °C. After 2 hours of incubation, samples were boiled for five minutes and then centrifuged to separate precipitated protein. The supernatants were analyzed by reverse phase HPLC (absorbance at 260 nm) on a Waters Sunfire™ C₁₈ 5 µm, 4.5 X 250 mm column. The running buffer
contained 100 mM NaH₂PO₄ pH 6.5, 10 mM Tetra-butyl ammonium bromide, 0.2 mM NaN₃, 7.5% Acetonitrile. Product elution times were compared to standards for GTP, GDP and GMP.

6.3.4 Crystallization, Data Collection and Model Building Single crystals of diffraction quality were grown by vapor diffusion from 5-12 mg/ml protein in 50 mM TRIS pH 7.5, 150 mM NaCl. The reservoir was mixed 1:1 with protein solution and contained 100 mM sodium citrate pH 4.8-5.4 and 0-5% PEG 5K MME. Native and Se-Met GsYqeH crystals belonged to the P2₁ space group with cell dimensions 47.6 Å X 81.1 Å X 108.2 Å Å and β=91.5°. Diffraction data for the native GsYqeH crystals (2.36 Å) were collected at CHESS beamlines F1 and F2 on a Q4 quantum CCD detector. SAD data (2.5 Å) for SeMet crystals were collected at the APS beamline NE-CAT 24-BM on a Q315 Quantum CCD detector. The datasets were reduced and scaled using HKL2000 (41). The GsYqeH model (resides 97-369) was built manually using XFIT (42) and COOT (43) in a 2.5 Å map generated from the SAD data by SOLVE and RESOLVE (44). A more complete model (GsYqeH residues 57-369) was then built and refined against the native dataset (2.36 Å) with AMoRe (45) and CNS (46). The structure was adjusted with XFIT and COOT to F(obs) - F(calc) and 2F(obs) - F(calc) maps. Addition of GDP and water molecules amidst cycles of refinement produced the final model (R: 27.1%, R(merge): 25.3%) (Supplementary Table 1).

6.3.5 Structure Analysis Programs and Computer Graphics Structural alignments of proteins / peptides were made with URAMS (47). Structural homology searches were carried out with DALI (48). The Function Site Prediction Server was used to identify functionally important residues, which were considered
significant if the generalized linear model score > 6.0 (49). Molscript (50) generated the molecular representations. The molecular representations were further enhanced using Adobe Photoshop.

6.3.6 Complementation of Atnoa1 by YqeH For constitutive expression of AtNOA1 in plants, the AtNOA1 ORF was amplified by PCR from a template consisting of AtNOA1 cDNA and digested with XbaI and SmaI. The PCR product was cloned into plant transformation vector pF3PZPY122 (51). This resulted in pF3PZPY122:AtNOA1, which encodes recombinant AtNOA1 tagged with three tandem FLAG epitopes at the C-terminus. A DNA fragment encoding the first 101 amino acids of AtNOA1 was amplified by PCR, digested with NheI and XbaI, and cloned into XbaI site of pF3PZPY122 to make pF3PZPY122:101AtNOA1. Then, the yqeH ORF was amplified by PCR, digested with XbaI, and ligated with pF3PZPY122:101AtNOA1 linearized by XbaI and SmaI, to generate the chimeric yqeH construct for the complementation experiment.

Agrobacterium strain LBA4404 was transformed with chimeric yqeH constructs and pF3PZPY122:AtNOA1, and the recombinants were screened in LB media containing chloramphenicol (10 μg/ml). Atnoa1 mutant plants were transformed by floral dip method (52). The transgenic plants were selected for gentamicin resistance (50 μg/ml Murashige-Skoog media) and allowed to set seeds. The genotypes of T2 plants were confirmed by PCR analysis of genomic DNA for the knock-out of wild-type AtNOA1, chimeric yqeH, and for constitutive expression of AtNOA1 cDNA.
6.4 RESULTS AND DISCUSSION

6.4.1 GTP Hydrolysis by GsYqeH Based on the sequence relationships to CPG domains and previous reports that B. subtilis YqeH could interact with and hydrolyze Guanine-nucleotides (31), we tested the ability of GsYqeH to bind and hydrolyze GTP. Initially, GsYqeH showed little evidence of nucleotide hydrolysis by HPLC analysis of products, but on addition of a known facilitator of nucleotide exchange (200 mM ammonium sulfate) (53) GsYqeH converts GTP to GDP over a period of hours in a Mg$^{2+}$-dependent manner (Figure 6.1). Similar findings were also made for AtNOA1 in a companion study that provides additional data for the GTP hydrolysis properties of this enzyme family (Moreau et al.).

![GTP hydrolysis by GsYqeH](image)

Figure 6.1: GTP hydrolysis by gsYqeH. HPLC profiles of GTP incubated with gsYqeH (blue) and GTP incubated without gsYqeH (red) reveal that gsYqeH is capable of catalyzing the hydrolysis of GTP to GDP in a Mg$^{2+}$-dependent manner. The identities of elution peaks were determined by running standards of GTP, GDP and GMP.

6.4.2 A Zinc Binding Motif in GsYqeH Sequence analysis and secondary structure prediction of YqeH homologs revealed an N-terminal motif found in the Treble-clef family of Zinc finger domains (24). The Treble-clef motif is
contained within diverse proteins whose functions range from the binding of nucleic acids, proteins and small molecules to the catalysis of phospho-diester bond hydrolysis (54). Inductively Coupled Plasma (ICP) analysis on GsYqeH, purified without addition of zinc, identified zinc as the most prevalent metal ion in the sample (data not shown). Zinc was present at nearly stoichiometric levels compared to protein. Zinc also affects the hydrodynamic properties of GsYqeH. Gel filtration chromatography (Superdex G75) of a GsYqeH sample prepared separately from the one analyzed above produced an elution profile with two closely separated peaks at a volume consistent with the predicted molecular weight of the protein (Figure 6.2). However, on addition of 300 μM zinc chloride (to 250 μM protein), the elution peak corresponding to the larger hydrodynamic radius disappeared and all of the protein appeared with the smaller hydrodynamic radius (Figure 6.2). Thus, a population of recombinant GsYqeH is deficient in zinc and zinc binding converts the protein to a more compact form.

Figure 6.2: Zinc binding by gSYqeH. Gel filtration profiles (Superdex 75) of gSYqeH (~41 KDa) incubated with (blue) and without (red) zinc chloride. Protein purified without added zinc elutes as a mixture of two species and addition of zinc compacts the protein, as visualized by a greater elution volume. The triple mutant C7S;C10S;C39S loses its ability to bind zinc.
(magenta) compared to native protein grown in the presence of excess zinc (cyan).

To identify the residues responsible for zinc binding, we sequentially mutated the conserved cysteine residues to serine in the Treble-clef domain. Single GsYqeH point mutants at C7S, C10S, or C39S, a double mutant C7S;C10S, and a triple mutant C7S;C10S;C39S were readily expressed and purified from E. coli. Proteins with the single and double substitutions eluted at the same position as zinc-bound native protein when expressed in rich media. However, the triple substitution C7S;C10S;C39S eluted as the zinc-free native protein and did not undergo any change in hydrodynamic radius when incubated with zinc (Figure 6.2). Note that the single and double Ser substitutions may also disrupt zinc binding, but a compact structure could result from disulfide bonding. Thus, the small N-terminal domain of YqeH is indeed a zinc-binding domain and is very unlikely to also contain the cofactors necessary to catalyze the conversion of Arg to NO.

6.4.3 Absence of NO Synthesis Activity Following previous reports on AtNOA1 (1), the Griess assay was employed in attempts to detect Arg conversion to NO and further oxidized species by YqeH. No NO production from GsYqeH was observed under any conditions (data not shown). These included, the presence and absence of the animal NOS cofactors tetrahydrobiopterin and calmodulin, and reductants supplied as peroxide or NADPH. Given that the structure of YqeH (see below) bears no resemblance to NOS and contains none of the NOS-essential cofactors or binding sites for them, it is consistent that the protein does not have NO synthase activity. Notably, in a companion study (Moreau et al.) extensive tests for NO synthesis activity and arginine binding
by AtNOA1 were also all negative.

6.4.4 The Crystallographic Structure of GsYqeH (CPG and C-terminal Domains):

6.4.4.1 Overall Structure Recombinant GsYqeH formed crystals that diffracted to 2.36 Å resolution. The structure (Figure 6.3) was determined by a single wavelength anomalous diffraction experiment on Se-methionine derivatized material (Table 6.1).

Figure 6.3: Crystal structure of GsYqeH. (A) The CPG-domain (right) has a central 7-stranded β-sheet surrounded by α-helices and binds GDP, which is exposed to the solvent on one side. The C-terminal domain (left) has a 2-fold pseudo-symmetric β-fold. (B) Alternate view of the C-terminal domain: The domain starts (bottom) with a 3₁₀-helix (dark orange), followed by an α-helix (purple), coil (purple), five β-strands (blue), a 3₁₀-helix (yellow), an α-helix (magenta), coil (magenta), and five β-strands (green).

The N-terminal zinc-binding domain (residues 1-58) is absent in the electron density maps, despite addition of excess ZnCl₂ during handling and no evidence for proteolysis in the crystals (as verified by SDS-PAGE). GDP that co-purified with the protein is found bound in the CPG domain. The CPG-domain has a typical G-protein fold with a central 7-stranded sheet (six parallel strands, one anti-parallel strand) surrounded by six α-helices (Figure...
6.3, 6.4 A). In G-proteins, five sequence regions termed G1-G5 in the order they appear along the protein sequence, play important roles in nucleotide exchange, GTP hydrolysis and conformational change (55, 56).

Table 6.1: Data collection and Refinement Statistics for gsYqeH

<table>
<thead>
<tr>
<th>Diffraction Statistics</th>
<th>Native gsYqeH</th>
<th>Se-Met gsYqeH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution (Å)</td>
<td>2.36 (2.44 – 2.36) ( ^{s} )</td>
<td>2.40 (2.49 – 2.40) ( ^{s} )</td>
</tr>
<tr>
<td>Number of unique reflections</td>
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<td>55226</td>
</tr>
<tr>
<td>Number of observations</td>
<td>255001</td>
<td>186671</td>
</tr>
<tr>
<td>% Completeness ( (I/ \sigma I)^{s} )</td>
<td>99.4</td>
<td>99.2</td>
</tr>
<tr>
<td>( R_{sym} ) (%)</td>
<td>7.6 (53.9) ( ^{s} )</td>
<td>15.7 (87.6) ( ^{s} )</td>
</tr>
</tbody>
</table>

SAD structure solution statistics

| Resolution cut-off (Å) | 2.50 |
| Number of Anomalous sites found | 13 (out of 18) |
| Mean figure of merit | 0.32 |
| Overall Z-score | 52.5 |

Refinement Statistics for Native gsYqeH

| Number of residues | 624 |
| Ligand | GDP |
| Number of water molecules | 467 |
| \( R^{c} \) (%) | 27.1 (39.6) \( ^{s} \) |
| \( R_{free} \) (%) | 25.3 (37.1) \( ^{s} \) |
| Overall \( B \) (Å\( ^{2} \)) | 49.9 |
| Mainchain \( B \) (Å\( ^{2} \)) | 45.1 |
| Sidechain \( B \) (Å\( ^{2} \)) | 55.9 |
| RMSD bonds (Å) | 0.009 |
| RMSD angles (degrees) | 1.9 |

\( ^{a} \) Intensity of the signal to noise ratio. \( ^{b} \) \( R_{sym} = \Sigma |I_{i} - \langle I \rangle| / \Sigma |I_{i}| \). \( ^{c} \) \( R = \Sigma |F_{obs} - |F_{calc}| / \Sigma |F_{obs}| \) for all reflections (no σ cutoff). \( ^{d} \) \( R_{free} \) calculated against 10% of reflections removed at random. \( ^{e} \) Overall model average thermal \( (B) \) factor. \( ^{f} \) Root mean square deviations from bond and angle restraints. \( ^{s} \) Highest resolution bin for compiling statistics.
G1-G5 are spatially close and are all associated with loop regions. G1-G4 are defined by conserved residues: G1 (GXXXGKS/T), G2 (T), G3 (DXXG), G4 (N/TKXD) (24, 56). Interestingly, in YqeH, the G-regions of the CPG domain are rearranged as G4-G5-G1-G2-G3 in the linear sequence (Figure 6.4 B).

Figure 6.4: Structure comparison of a canonical versus a circularly permuted GTPase. (A) Structural superposition of GDP bound forms of the GsYqeH CPG-domain (green) and a canonical small G-protein Cdc42 (blue). Four out of the five G-regions (except G2) overlap almost exactly, though their order in the linear sequence is permuted with respect to each other. There is a long coiled region between $\alpha_4$ and $\beta_2$ in Cdc42, but in GsYqeH, the corresponding region continues as a helix, making $\alpha_{16}$ (GsYqeH) longer than $\alpha_4$ (Cdc42). In GsYqeH, $\alpha_{16}$ continues as $\beta_{15}$ before it joins to $\beta_{16}$ in GsYqeH, which corresponds to $\beta_2$ in Cdc42. (B) Comparison of GsYqeH CPG domain and Cdc42 topologies. In the structural alignment, $\alpha_{11}$ in GsYqeH corresponds to the two $3_{10}$ helices in Cdc42 directly below it. $\beta_{16}$ corresponds to $\beta_2$, $\beta_{17}$ to $\beta_3$, etc. Cdc42 $\alpha_4$ and the preceding $3_{10}$ helix are absent in GsYqeH.

However, this permutation allows for the same 3D fold observed in canonical GTPases such as the small G protein Cdc42 (1ANO.pdb) (Figure 6.4 A) and the nucleotide-binding domains of larger G-proteins such as Transducin (1GG2.pdb). This same permutation is also exhibited by the CPG domains of proteins such as YlqF from B. subtilis (1PUJ.pdb), YjeQ from Thermotoga maritima (1U0L.pdb), and YloQ from B. subtilis (1T9H.pdb). In GsYqeH, four of
the five G-regions, except for G2 (Figure 6.4 A), overlap almost exactly with the same regions in GDP-bound Cdc42. The GsYqeH sequence permutation is produced by breaking the loop connecting $\beta_3$ and the $3_{10}$ helix in Cdc42 to generate the N- and C-termini of the domain, and then connecting $\beta_{14}$ to $\alpha_{15}$, which correspond to the N-termini ($\beta_1$) and C-termini ($\alpha_5$) of Cdc42, respectively (Figure 6.4 B).

6.4.4.2 Guanine Nucleotide Binding The bound nucleotide (GDP) in GsYqeH is surprisingly exposed to solvent compared to that of other G-proteins. The $\alpha, \beta$ phosphates of GDP interact with the so-called “P-loop” which is located between $\beta_{14}$ and $\alpha_{16}$ (G1). Other regions in close contact with the GDP are the turns between $\alpha_{21}$ and $\beta_{21}$; $\beta_{12}$ and $\alpha_{13}$ (G4); $\beta_{13}$ and $\alpha_{15}$ (G5), and the extended coil containing the G3 region, which is also known as Switch II (55). The protein provides multiple interactions to the bound nucleotide. Asp-109 hydrogen bonds to N2 and N3 of the guanine base and the entire side chain of Lys-107 forms an aliphatic “bed” that upon the guanine ring lies. The hydroxyl group and backbone nitrogen of Ser-176 hydrogen bond to the $\beta$-phosphate whereas the backbone nitrogen of Thr-177 hydrogen bonds to the $\alpha$-phosphate. An ordered water molecule hydrogen bonds to both phosphates on the solvent-exposed side and to the carbonyl oxygen of Asn-172. GDP-bound YqeH does not provide any interactions for the hydroxyl groups of the ribose sugar ring.

6.4.4.3 GTP Binding and Hydrolysis Require Movement of Switch I Regions of G-proteins called Switch I and II, which usually comprise G2 and G3 respectively, undergo conformational changes that trigger recognition of
binding partners when G-proteins exchange GDP for GTP (55). In YqeH, the G2 region has atypical structure and is remote from the nucleotide. In contrast, G1 of Cdc42 is followed by $\alpha_1$ which leads into a long coiled region that contains G2 and closely juxtaposes the bound GDP. In GsYqeH, the $\alpha_1$-analog, $\alpha_{16}$, does not connect to a G2-containing coil region, but rather extends into a longer helix (Figure 6.4 A). The longer $\alpha_{16}$ continues into $\beta_{15}$ before connecting to $\beta_{16}$, which corresponds to $\beta_2$ of Cdc42. Nevertheless, G2 of GsYqeH contains a conserved threonine residue (Thr-202) that participates in the hydrolysis mechanism of other GTPases. Involvement of YqeH Thr-202 in GTP hydrolysis would require a large rearrangement of G2 and regions surrounding Switch I. Interestingly, a similar movement of G2 has been observed in the Ran GTPases. Ran plays a vital role in the nuclear transport machinery (57, 58), where GTP hydrolysis regulates Ran-mediated interactions between importin-β-like transport receptors and diverse cargo such as such as histones (59), snRNPs (60, 61), hnRNPs (62, 63), mRNA binding proteins (64, 65), and tRNA (66, 67). In GDP-bound Ran, the G2 region that contains the catalytic Thr-42 (Thr-202 in GsYqeH) lies in a loop region, far from the nucleotide. (Figure 6.5 A, 6.5 B). GTP binding restructures G2 and allows Thr-42 to engage the $\beta$ and $\gamma$ nucleotide phosphates (Figure 6.5 B) (68, 69).

Mutation of two conserved Thr residues in YqeH G2 indicates that Switch I likely undergoes a similar conformational change to that observed for Ran. T201A has much less GTP hydrolysis activity compared to native GsYqeH, but T202A is completely inactive (Figure 3C). In the Ran system, a family of Ran-binding domains (e.g. RanBP1) stabilizes the GTP-bound
conformation of Switch I and assists in nucleotide hydrolysis (70, 71). Additional factors capable of playing a similar role could act as activators of YqeH GTPase activity.

Figure 6.5: Structure comparison of the GDP bound form of YqeH and the small GTPase Ran in GDP and GTP bound forms. (A) Switch I region (Thr-202) (yellow) in GsYqeH-GDP is located far from the active site. (B) In Ran, conformational change in Switch I (Thr-42) brings the catalytically important residue close to the phosphates of GTP (blue), versus the GDP bound form (yellow). (C) T202A substitution in YqeH completely abolishes GTPase activity, consistent with this residue moving into the active site on GTP-binding. Assays were run in triplicate for 2 hours.

6.4.4.4 GD(T)P Exchange may Position the C-terminal Domain via Switch II Structural comparisons between YqeH and YlqF indicate that on nucleotide exchange Switch II (G3) of GsYqeH likely undergoes conformational changes that re-position the YqeH C-terminal domain. Both YlqF and YqeH belong to the CPG and HAS subfamilies of GTPases and have been implicated in B. subtilis ribosomal subunit assembly (28, 29, 33). In YqeH and YlqF, an Ile residue (222 in YqeH) of G3 substitutes for the catalytically important Gln / His found in many other GTPases. The crystal structure of B. subtilis YlqF bound to a non-hydrolysable GTP analog (GMPPNP) (New York Structural
GenomiX Research Consortium; PDB ID: 1PU]), reveals a C-terminal domain that is unlike that of YqeH (Figure 6.6), but a CPG domain that is very similar.

![Diagram A](image1.png)

![Diagram B](image2.png)

Figure 6.6: GTP hydrolysis may change orientation of C-terminal domain. Superpositions of the CPG-domains of GsYqeH (blue) and BsYlqF (pink) with the C-terminal domains of either BsYlqF (A) or GsYqeH (B) removed for clarity. Comparison of GTP-bound YlqF and GDP bound YqeH indicates that GTP binding displaces the conserved Ile from the phosphate pocket and thereby reorients the C-terminal domain.

Furthermore, the G3 regions that connect the CPG to the C-terminal domains in both GsYqeH and BsYlqF are closely related in sequence (LYDTPGII in GsYqeH versus LLDTPGIL in BsYlqF) and both retain an Ile residue that resides next to the nucleotide phosphates. However, the conserved G3 regions have different conformations in the two proteins due to the presence of GDP in YqeH and a GTP analog in YlqF. In YlqF, the Ile side chain points away from the nucleotide, whereas in YqeH, the Ile-222 side chain flips into the pocket that would be occupied by -phosphate in YlqF (Figure 6.6). It follows that GTP uptake by YqeH will displace Ile-222 and G3 will obtain a conformation similar to that found in GTP-YlqF. The resulting movement in
the G3 linker would reposition the C-terminal domain (Figure 6.6). This may be the switch conferred by GTP hydrolysis to switch the protein between different states of activity.

6.4.4.5 The YqeH C-terminal Domain The C-terminal domain (residues 226-369) has a novel pseudo-two-fold symmetric-sheet topology (Figure 6.3 B). The N- and C-terminal halves of the dyad are hereafter referred to as C_N and C_C. Both C_N and C_C contain a 3_{10}-helix, an α-helix and five β-strands and are related by a two-fold axis roughly parallel to the β-strands. C_N and C_C have low sequence identity (14%) but high structural similarity (C_α RMSD: 2.1 Å for 55 out of 70 residues) (Figure 6.7 B, 6.7 D).

Figure 6.7: The Peptide-Nucleotide-Recognition (PNR) fold. The β-sandwich fold common to YqeH, TRAP and pVHL contains five β-strands (β_1-β_5) and interacts with both peptides and nucleic acids. Shown in the above figure are: (A) BsTRAP residues 8-58; (B) GsYqeH residues 318-365 (C_C); (C) human pVHL residues 73-121; (D) GsYqeH residues 246-290 (C_N). All of the above structures share structural similarity with the same topologies and C_α-RMSDs of 2.0-2.8 Å. In bsTRAP residues Asn-20, Phe-32 and Arg-58 are involved in RNA binding. Analogous residues in GsYqeH are predicted to be of functional importance. pVHL also contains the Arg residue common to TRAP and YqeH. β_3 and β_4 in pVHL and in YqeH participate in peptide binding, β_4 and β_5 in TRAP participate in peptide binding. Analogous to TRAP, the solvent exposed surface in YqeH formed by the strands β_2, β_3 and β_4 may bind RNA.
6.4.4.6 Structural Homologs of Functional Relevance A structural homolog search using DALI (48) returned no significant homologs when the C-terminal domain was queried against the entire PDB (Z-score ≤ 2.0), indicating that as a whole, this domain has a novel fold. However, the inherent two-fold symmetry of the domain suggested that one half of the dyad may represent a more fundamental folding unit (Figure 6.7 B). A search with this unit, Cc (residues 291-369) revealed a structural relationship to the protein TRAP (Trp RNA-binding Attenuating Protein) from B. subtilis (Z-score = 4.0; length of match: 43 residues, C RMSD: 2.0 Å; Figure 6.7 A, 6.7 B). When complexed with L-tryptophan, TRAP binds to the leader regions (11 G/UAG repeats) of the mRNA encoding the Trp biosynthetic enzymes, thereby inhibiting their translation (72, 73). RNA base triplets (G/UAG) wrap around the TRAP oligomer, which composes a ring of 11 subunits. The C-terminal domain of YqeH most likely does not bind Trp as none of the TRAP Trp-binding residues are conserved in YqeH. Furthermore, due to the pseudo symmetry within the dyad, the C-terminal domain of YqeH cannot oligomerize like the TRAP molecules. Nonetheless, there are interesting correlations between the surface properties of the two proteins with respect to the function of TRAP. The YqeH C-terminal domain conserves some residues that have been shown to be important for RNA binding by TRAP (73). An invariant TRAP Arg-58 that binds G/UAG tri-nucleotide repeats aligns exactly with invariant Arg-365 in GsYqeH (Figure 6.7 A, 6.7 B). In both structures, these residues are completely exposed and appear to have no important function in stabilizing the structures of the respective domains.
A highly conserved Asn residue (Asn-20) in TRAP that lies beside Arg-58, aligns well with a conserved aspartate (Asp-334) in GsYqeH (Figure 6.7 A, 6.7 B). A conserved Phe-32 in TRAP that -stacks with the latter G in the G/UAG repeats mirrors conserved Trp-342 of GsYqeH (Figure 6.7 A, 6.7 B). The conserved Arg-365, Asn-334, Trp-342 triad of GsYqeH was also identified by a method independent of the TRAP relationship. Analysis of the YqeH structure with the “Function Site Prediction Server” (49), which filters sequence conservation due to functional relevance from conservation due only to structural constraints, also identified Arg-365, Asn-334, and Trp-342 as the highest scoring residues for functional relevance in Cc, along with a symmetry related triad on Cn (Phe-260, Arg-266, Asp-268).

6.4.4.7 Structural Homology to pVHL The DALI search with Cc also revealed homology to the Von-Hippel-Lindau tumor repressor protein (pVHL) (Z-score: 3.9). pVHL is a regulator of the hypoxia-inducible transcription factor (HIF-α), that activates genes involved in angiogenesis, apoptosis, and proliferation in response to hypoxia conditions (74, 75). pVHL binds to HIF-α when a critical HIF-α proline is hydroxylated by an oxygen requiring prolyl-4-hydroxylase. pVHL then forms a ubiquitin-protein ligase complex along with elongin B, elongin C, Cul2 and Rbx1 that targets HIF-α for degradation (74, 75). Despite very low sequence similarity, Cn or Cc of YqeH have structural similarity to the -domain of pVHL that recognizes the hydroxyprolyl-peptide (Figure 6.7 B, 6.7 C, 6.7 D). Of the GsYqeH triads, only Arg-365 has an analog in Arg-120 of pVHL, (Figure 6.7 B, 6.7 C). Arg-120 is well conserved in the pVHL sequences, but is not important for peptide recognition. In addition, none of the residues that are responsible for binding the hydroxylated peptide
in pVHL are conserved by YqeH.

The Function Site Prediction Server identified three residue clusters on the surface of pVHL important for function. One cluster (Trp-88, Tyr-98, Leu-101, Pro-103, His-115, and Trp-117) includes those residues involved in binding hydroxylated HIF-1. This region has been implicated in pVHL self-association (76), as well as the binding and inhibition of the transcriptional activator Sp1, which stimulates transcription of the hypoxia-related factors VEGF and GLUT-1 (76). Another conserved cluster (Leu-158, Leu-163, Ile-180 and Leu-184) comprises those residues important for elongin C binding. The third cluster (Arg-82, Phe-119, Asp-121, Val-130, Ile-151) contains residues positioned on a face of the molecule similar to where the clusters composing the TRAP/YqeH triad reside. Although the sequence positions of the Arg, Phe and Asp residues are different from those in TRAP and YqeH, the similar composition of the triad in the three proteins may reflect a common RNA-binding function. There is some evidence that pVHL may destabilize certain mRNA, although the mechanism of action is not well understood (77-82).

6.4.4.8 The Peptide-Nucleotide Recognition (PNR) Fold The structural module common among YqeH, pVHL, TRAP and AtNOA1 appears to be an adaptor capable of making interactions between proteins and nucleic acids. Within this β-sandwich topology, β₁ and β₄ form one sheet, and β₂ β₃ and β₅ form the other (Figure 6.7). TRAP and pVHL interact with polypeptides through residues on β₄ β₅ and β₂ β₃ respectively. In TRAP the interaction is self-oligomerization (72); in pVHL, the interaction involves recognition of Sp1 (76) and hydroxylated HIF1-α (74, 75) and perhaps self-oligomerization (76).
Analogous regions in the YqeH C-terminal domain also participate in polypeptide binding in the sense that they recognize each other, with $\beta_3$ of $C_N$ binding $\beta_4$ of $C_C$ and visa versa.

This module also binds RNA and hence we refer to it as a peptide/nucleotide recognition (PNR) domain. In TRAP, the solvent-exposed surface of the -sheet formed by $\beta_2$, $\beta_3$, and $\beta_5$ binds RNA and contains the triad Asn-20, Phe-32, and Arg-58. Similar residues on the analogous solvent-exposed surfaces of YqeH $C_N$ and $C_C$ may also bind RNA. In YqeH and AtNOA1, GT(D)P exchange may be functionally linked to RNA binding. In the GDP-bound GsYqeH, the predicted RNA-binding triad (Asp-334, Trp-342 and Arg-365) is shielded from solvent by the CPG domain. However, if exchange of GTP alters the connection between the CPG and PNR domains (Switch II) to a conformation similar to that observed in the GTP bound form of Y1qF (Figure 6.6), these residues will become exposed and hence their ability to bind targets (e.g. RNA) will be enhanced. The YqeH target may also activate YqeH by restructuring Switch I into a conformation appropriate for GTP hydrolysis. Given these analogies, it is interesting that the hydrolysis mechanism of the YqeH CPG domain is related to the mRNA/protein localization regulator Ran.

6.4.5 Complementation of Atnoa1 by Chimeric YqeH

Compared to wild-type, Atnoa1 mutant plants showed growth retardation and developed yellowish rosette leaves (1, 83) (Figure 6.8 A, 6.8 B). The latter defect was rescued by application of NO donors (1), which suggested a relationship between the morphological defect and NO deficiency in the
mutant plants. To test whether YqeH is an ortholog of AtNOA1, the bacterial protein was expressed in the *Arabidopsis Atnoa1* mutant plants.

![Images of plants](image)

Figure 6.8: Complementation of *Atnoa1* with chimeric *yqeH*. (A) Wild-type, (B) *Atnoa1*, (C) T2 generation of *Atnoa1* transformed with chimeric *yqeH*, and (D) T3 generation of *Atnoa1* transformed with wild type AtNOA1. The seed germination was synchronized by cold treatment. Photography was done two weeks after germination. The size bars correspond to 3 mm. Arrowheads point to emerging rosette leaves.

To facilitate proper targeting of YqeH in plants, a chimeric *yqeH* construct, which encodes GsYqeH with the first 101 amino acids of AtNOA1 fused to its N-terminus, was used for the plant transformation. The chimeric YqeH complemented the morphological defects of *Atnoa1* as efficiently as wild-type AtNOA1 (Figure 6.8 C, 6.8 D). Both transgenic plants developed green rosette leaves, and their growth was comparable to that of wild-type plants. It has been recently reported that *B. subtilis* YqeH will also complement the growth and coloration phenotypes of *Atnoa1* mutant plants when it is fused to a peptide that targets it specifically to plastids / chloroplasts (84). This study further showed that native AtNOA1 is imported into chloroplasts both in leaves and *in vitro* (84). This provides an interesting contrast to the supposition that the leader peptide of AtNOA1 is for mitochondrial targeting (84) and supporting studies that found AtNOA1 in the mitochondria of roots (84).
Thus, AtNOA1 may be important for the function of both prokaryotic derived organelles: plastids and mitochondria.

6.5 Full circle: Implications of GsYqeH for the structure and function of AtNOA1

The overall structure of AtNOA1 should be very similar to the structure of GsYqeH. Excluding the N-terminal leader peptide (83), the remaining ~100 extra residues in AtNOA1 as compared to the bacterial homologs occur as insertions in loop and -turn regions of the CPG and C-terminal domains (Figure 6.9).

Figure 6.9: Sequence alignment of AtNOA1 (A) and YqeH (Y). $\alpha_1$-helices and $\alpha$-helices are denoted by cylinders and $\beta$-strands by thick lines with arrows. The protein is divided into three domains: ZBD (Zinc Binding Domain), CPG (Circularly Permutated G-domain) and CTD (C-Terminal Domain). The residues that define the Treble-clef zinc site are shown in yellow and the guanine nucleotide binding regions G1-G5 in green. The insertions in the AtNOS1 protein sequence, as compared to the bacterial homolog (magenta), occur only in $\beta$-turn andloop regions.

Given the complementation data, we expect that AtNOA1 also acts as a switch protein that couples GTP hydrolysis to nucleic acid and / or protein binding. Recognition of ribosome components in plant mitochondria and / or plastids
may be one of AtNOA1's functions, given that depletion of YqeH leads to a
defect in ribosome biogenesis in B. subtilis. How this would affect NO
metabolism is not obvious (Moreau et al.), although it is worth noting that NO
availability is closely linked to the concentration of oxygen radicals, of which
the mitochondria and chloroplasts are major sources. Separate studies now
show localization of AtNOA1 to both mitochondria (84) and chloroplasts /
plastids (84). Further work aimed at linking AtNOA1 function to NO
availability, as influenced by mitochondrial and chloroplasts / plastids
associated processes, may prove fruitful.
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