

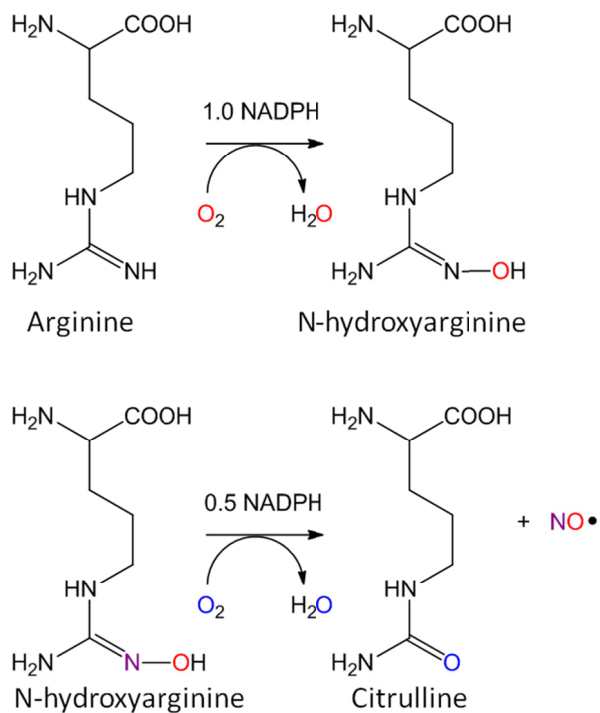
Chapter 1

Introduction and Background

1.1 Nitric Oxide Synthases

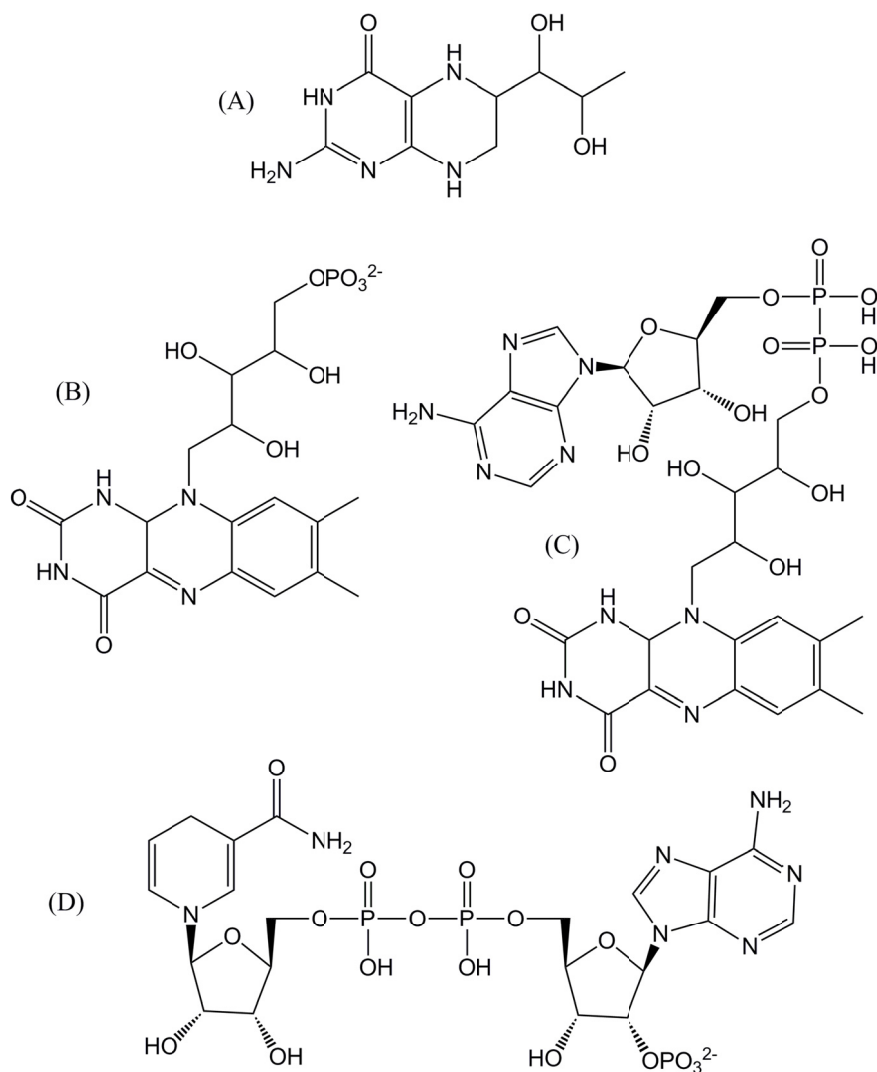
Salvador Moncada and colleagues reported in 1987 that the molecule responsible for relaxation of blood vessels is nitric oxide (NO).¹ This publication marked the beginning of a new area of chemical and biological research, now with thousands of articles published each year. Long known as a cytotoxic agent in pathological processes and a major component of smog, NO is now recognized as a key signaling molecule in the cardiovascular, immune, and nervous systems.²

Nitric oxide synthases (NOSs) are responsible for the production of NO in living systems.³ The three (mammalian) isoforms of the enzyme are named for the tissues in which they are found: endothelial NOS (eNOS), neuronal (nNOS), and an inducible form found in macrophages (iNOS).⁴ NOS enzymes have been identified in some bacterial species as well, such as *Bacillus subtilis* and *Sorangium cellulosum*.⁵⁻⁶ NOS catalyzes the oxidation of L-arginine (Arg) to L-citrulline in two turnovers, with N-hydroxy-L-arginine (NOHA) as an enzyme-bound intermediate (the product of the first turnover). The overall reaction is shown in **Scheme 1.1**.



Scheme 1.1. Production of NO by nitric oxide synthases from the starting material arginine.

Each mammalian enzyme forms a homodimer of two identical polypeptide chains.⁷ Each single, long (1000+ residue) chain contains a reductase domain, a calmodulin linker, and an oxygenase domain (**Figure 1.1**). The reductase domain contains binding sites for NADPH (nicotinamide adenine dinucleotide phosphate) and the flavins FMN (flavin mononucleotide or riboflavin 5'-phosphate) and FAD (flavin adenine dinucleotide), all cofactors are shown in **Scheme 1.2**.⁸ The calmodulin linker is a calcium responsive agent that causes structural changes in the presence/absence of Ca²⁺. The oxygenase domain binds the substrates (arginine and N-hydroxy-L-arginine), a heme cofactor, and a redox-active tetrahydrobiopterin cofactor. It is at the heme cofactor in the oxygenase domain where oxidation of substrate occurs.



Scheme 1.2. Cofactors involved in electron transfer in NOS: (A) tetrahydrobiopterin, (B) FMN, (C) FAD, and (D) NADPH.⁹

The mammalian isoforms are regulated through a complicated system of checks and balances. The functions of eNOS and nNOS are regulated by calcium ions and a calmodulin linker, while iNOS is calcium ion independent.¹⁰ Calmodulin is a polypeptide chain, in this case literally fused to the chain of the NOS enzyme, and is very sensitive to the presence of calcium regulating eNOS and nNOS by preventing electron transfer to the oxygenase domain in the absence of Ca.¹¹ On the other hand, the levels of expression of inducible NOS is regulated very carefully within white blood cells.¹⁰ Further, the

reduction potential of iNOS is also controlled by the presence of substrate in order to prevent release of reactive oxygen species. (When Fe(II) is produced in the absence of substrate, oxygen binds, oxidizes the iron to Fe(III), and is released as superoxide.)¹²

The oxygenase domain (NOSoxy) contains a thiolate-ligated heme (protoporphyrin IX, or P-IX) as in cytochromes P450 (P450) and (6R)-5,6,7,8-tetrahydrobiopterin (pterin, H₄B). NO is produced when this domain is supplied with electrons from the reductase domain to activate dioxygen, in the presence of fully reduced pterin cofactor. In the absence of H₄B no NO is produced, but rather other NO_x species.¹³ Although structural characterization of full-length NOS has not been reported to date, structures of individual domains are known.¹⁴⁻¹⁵

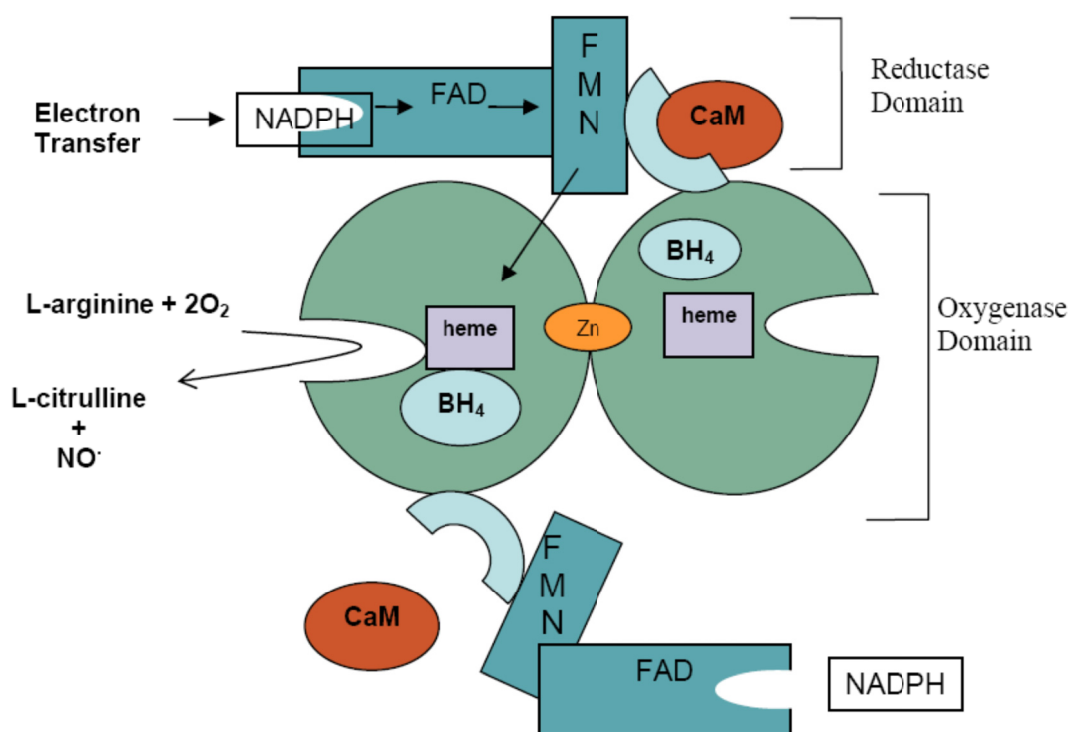


Figure 1.1. Graphical representation of the domains and geometry of nitric oxide synthases. (YHL Nguyen, PhD thesis from Caltech). (Note: No crystal structure of any full-length mammalian nitric oxide synthase has ever been reported, only the separate domains, thus it is still unclear how and where the two domains interact.)

1.2 Mechanism of NO Production

Arginine is oxidized to nitric oxide in two full turnovers, through the intermediate N-hydroxy-L-arginine. The intermediate actually has a higher binding affinity than arginine, preventing it from leaving the binding pocket, where it is positioned above the heme.¹⁶ The first turnover is a two-electron oxidation of substrate, formally a hydroxylation of one of the guanidinium nitrogens.² The stoichiometry of this reaction is identical to that of hydroxylations carried out by the extensively-studied cytochromes P450.¹⁷⁻¹⁸ The second reaction, however, is unique in biology. The use of the tetrahydrobiopterin in a redox-active manner (**Scheme 1.3**) is unique to NOS. Also, the second turnover is formally a three-electron oxidation of NOHA to citrulline and NO, specifically the radical species and not any other nitrogen oxide.³

The mechanism of NO production is not completely understood. The resting state of the enzyme is a six-coordinate ferric heme with a water molecule occupying the sixth ligand position (four positions are occupied by N donors from the porphyrin and one by a sulfur atom from an axial cysteine, Cys194).⁸ Although neither Arg nor NOHA ligates the heme, substrate binding shifts both the Soret absorption maximum and the heme spin state. The presence of substrate in the binding pocket sterically excludes water, forcing a high-spin five-coordinate heme complex.¹⁸ One-electron reduction of the NOS:substrate complex gives a five-coordinate ferrous heme that readily binds dioxygen, forming a ferrous-oxy species (equivalent to ferric superoxide), the last observed intermediate in the catalytic cycle.¹⁹

The role of pterin has been extensively investigated. This molecule binds in a pocket alongside the heme, forming a hydrogen bond with a protoporphyrin-IX carboxylate, thereby coupling it to the active site.²⁰ It is known that a pterin-based radical forms and is reduced during the catalytic cycle, as determined by analysis of results from rapid-freeze EPR experiments.²¹⁻²³ Production of NO has never been observed without fully reduced pterin cofactor, such conditions produce cyano-ornithine and nitrite rather than citrulline and NO.¹³

The NOS reaction cycle bears many similarities to that of cytochromes P450 (cyt. P450). Cyt. P450s contain thiolate-heme active sites and hydroxylate substrates via two-electron oxidation processes.¹⁸ The cyt. P450 cycle also begins with substrate binding followed by heme reduction, dioxygen binding, and another reduction step leading to the formation of a high-valent iron-oxo complex (Compound I) that hydroxylates the substrate (**Scheme 1.3**). Separate enzymes serve as reductases for most cyt. P450s, but substrate hydroxylation can be driven using external sources of electrons.¹⁷ It is of note that one cytochrome P450 has been found with an attached reductase domain: cyt. P450 BM3.²⁴ The reductase domain of this enzyme also shuttles electrons from NADPH through two flavins to the heme cofactor just like mammalian NOS, although it does not need to dimerize to function as NOS does. Owing to these similarities, the mechanism of the first turnover of NOS is postulated to be the same as that of cyt. P450s. However, the second turnover, a three-electron oxidation, is thought to employ a unique mechanism.²⁵ It has been suggested that a protonated ferric hydroperoxide may act as the nucleophile in the second turnover rather than Compound I, which is a ferryl P-IX radical cation.¹⁶

Another similarity between cyt. P450s and NOSs is the presence of three universally-conserved hydrogen bond donors to the axial thiolate ligand. All three come from backbone amide groups in P450, but in NOS only two come from amides, the third donor being the N-H group on a tryptophan residue.²⁶⁻²⁷ This Trp is conserved in every NOS identified to date.²⁸ In P450, there is a phenylalanine in that position which π -stacks with the porphyrin ring, just as this Trp does, although it is in no way involved in hydrogen bonding. The role of these hydrogen bond donors and π -stacking in tuning the electronics of the thiolate donor and porphyrin ring has not previously been fully investigated. The fact that these donors come from amides in the backbone make

mutagenesis nearly impossible (one was replaced by a proline residue, removing the amide group, but also shifting an entire loop within the protein, confusing results).

Steps in the mechanistic cycle borrowed from cyt. P450 are shown in **Scheme 1.3**. Although several intermediates in the cyt. P450 cycle already have been observed, there can be no doubt that “the hunt for an unambiguous experimental identification of the ephemeral active oxygen species will most certainly continue”.²⁹ In fact, after several decades of research on cyt. P450s it was only within the past year that the elusive, high-valent Compound I was positively characterized and shown to be the active hydroxylating oxidant.³⁰ If that is the case for cyt. P450, then we may conclude that work on the NOS catalytic cycle is just beginning.

1.3 Bacterial Nitric Oxide Synthases

The function of inducible nitric oxide synthase in mammalian macrophage cells is predominantly to kill the cells of invading bacteria by pumping them full of nitric oxide.¹⁰ NO is a radical species and therefore reacts rapidly with many parts of cells causing extensive damage. Given its usefulness in killing bacterial cells, it was surprising when researchers discovered NO synthase-like proteins in prokaryotic systems in the early 2000s.³¹⁻³² Since then, NOS-like proteins have been identified in all kingdoms of life, with examples in archaea and bacteria, emphasizing their biological importance.⁵ Their presence in several pathogenic species is of particular interest. The bacterial NO synthases from three phyla of Gram-positive bacteria (actinobacter, deinococcus, and firmicutes) in particular share high levels of homology with the oxygenase domains of eukaryotic enzymes.⁶

Bacterial enzymes share many similarities with their mammalian counterparts. First, they share surprising sequence homology, with about 45% of their peptide sequences being identical and 50–60% similar.³³ Comparison of their overall three-dimensional folds reveals strikingly similarities (**Figure 1.2**); nearly every helix and loop is mirrored in each system studied to date.⁵ All important residues are maintained, those involved in hydrogen bonds with the substrate and cofactors, and the cysteine providing iron ligation, to highlight a few. All NOS oxygenase domains, regardless of species of origin, contain binding sites for the heme cofactor (protoporphyrin-IX), substrate, and the necessary pterin. All of these overlay exactly when aligning multiple structures, if one simply centers the structures around the iron atom in the heme.^{32, 34} Every NOS requires substrate, both cofactors, reducing equivalents and dioxygen to function. When these pieces are combined, NO is produced catalytically.

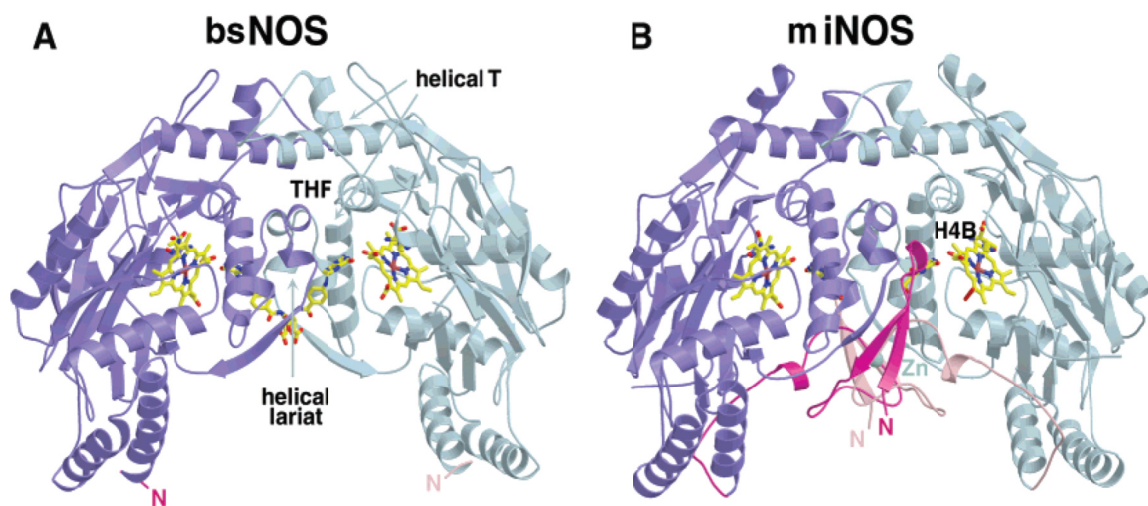


Figure 1.2. A crystallographic comparison of the three-dimensional folds of a bacterial NOS (A, from *Bacillus subtilis*) and a mammalian NOS (B, from inducible NOS found in mammalian macrophages). Note that all NOS oxygenase domains crystallize as a dimer. The missing N-terminal Zn-binding loop is highlighted in fuchsia, B.³²

There are also, however, some striking differences between bacterial and eukaryotic systems. The largest difference is that only one bacterial NOS (bNOS) has been identified to date that contains a fused reductase domain within its amino acid sequence.³⁵ Nearly all bNOS enzymes are made up of only the oxygenase domain where the chemistry of NO production occurs (the NOS from *Sorangium cellulosum* being the only exception). This raises the question of how reducing equivalents can be delivered. bNOS is also missing a zinc-binding loop contained in all the mammalian isoforms. This loop is necessary for the dimerization of mammalian systems, a requirement for function because a reductase domain from one monomer of enzyme provides the reducing equivalents for the oxygenase domain of the other monomer.³⁶ Truncation of the peptide chain in order to remove this loop results in the abolishment of catalytic activity. This loop partially obstructs the pterin cofactor binding site, protecting it from solvent.³⁷ Some bacteria cannot synthesize tetrahydrobiopterin, they simply lack the necessary sequences in their genomes.⁶ It has been proposed that removal of this loop allows room to accommodate the larger pterin, tetrahydrofolate, which all these bacteria are able to synthesize.⁵ The final major difference is a single point mutation near the heme. This position is a conserved valine among eukaryotic systems, while in bacteria it is an isoleucine.³⁸ It has been shown previously that this residue, situated right above the iron atom and within Van der Waals contact distance of Fe-NO species (Ile), affects the rate of NO release from the enzyme.³⁹ These differences may highlight key functional differences among species.

Given these few but striking differences, it was necessary to prove that bacterial NO synthase-like proteins did in fact produce nitric oxide, and using the same chemistry

as their eukaryotic counterparts. One landmark study required the collaboration of three groups, those of Stephen Lippard, Dennis Stuehr and Evgeny Nudler.³³ It is a complicated process to definitively prove that an enzyme functions and produces NO, not any other species, *in vivo*. In oxygenated aqueous solution, NO is oxidized rapidly to nitrite and nitrate. Reagents have been developed that can colorimetrically detect these NO metabolites in solution (Griess Assay, Cayman Chemicals). As NO transforms to NO_2^- and NO_3^- in solution, the concentration of these in solution is proportional to the amount of NO produced.³⁴ This team of researchers used both the Griess Assay to detect NO in the extracellular environment of the cells of *B. subtilis* and *B. anthracis* and an NO-specific fluorescent probe called CuFL that allows for intracellular NO detection. These techniques, in combination with creative use of an arabinose promoter, allowed them to prove that NO is indeed produced in these cells by their NOS enzymes.

The demonstration of NO synthesis within bacterial cells raises the question of why NO is produced.⁶ The signaling functions of NO in eukaryotic systems are mediated by the NO receptor, soluble guanylate cyclase (sGC).⁴⁰ A bacterial homolog of sGC has been identified as a family of H-NOX proteins found by Michael Marletta and coworkers.⁴¹ Interestingly, though, no H-NOX protein has been found in the genome of any bacteria that also code for NOS.⁴² No other NO receptors have been identified. It has been proposed that in pathogenic bacteria the synthesis of NO promotes resistance to oxidative stress caused by the host immune system.⁴³ NO may also promote antibiotic resistance, due to its ability to chemically modify many compounds used as antibacterial agents.⁴⁴ While these hypotheses may explain the role of NO in pathogenic strains such

as *Staphylococcus aureus* and *Bacillus anthracis*, the role of NO in non-pathogenic bacteria remains a mystery.

The NOS (gsNOS) from a non-pathogenic bacterial thermophile, *Geobacillus stearothermophilus*, is the focus of this majority of this work. Only one chapter deals with the mammalian inducible isoform, the rest focus on this unique bacterial enzyme. gsNOS is noted for the particularly stable ferrous-oxy complex it forms.³⁴ This complex lasts only a few seconds at most in other enzymes, but is stable on the order of a minute in gsNOS at 4 °C. It is not incredibly surprising that the kinetics of this enzyme are slower at standard temperatures than other enzymes, given it comes from a thermophilic organism and must function properly at significantly elevated temperatures. It is this stability that makes this a useful system to study. This enzyme was originally expressed, characterized, and crystallized by Brian Crane and coworkers at Cornell.³⁴ The protein fold as revealed by X-ray crystallography is shown in **Figure 1.3**, with a close-up on the heme-thiolate active site.

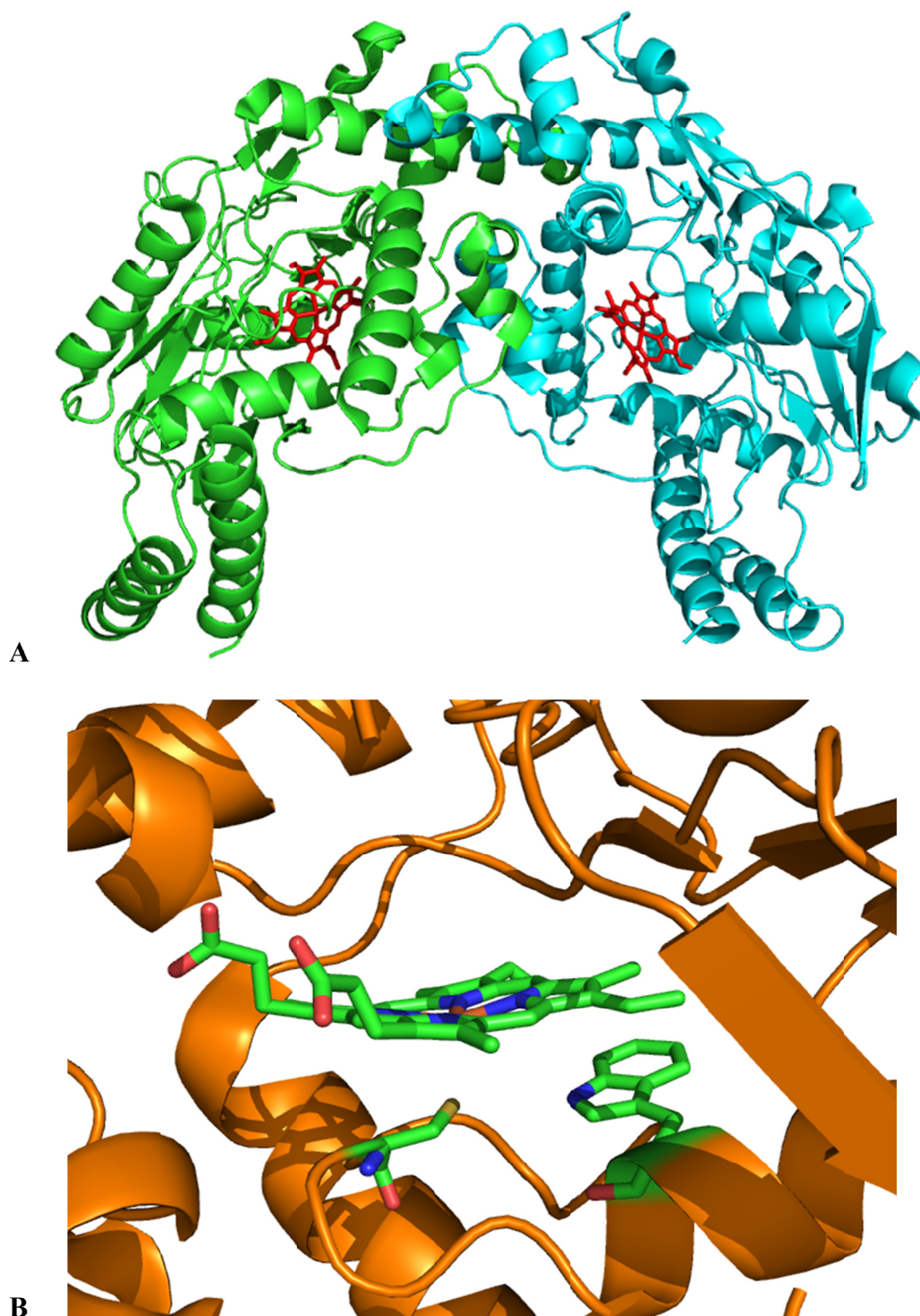


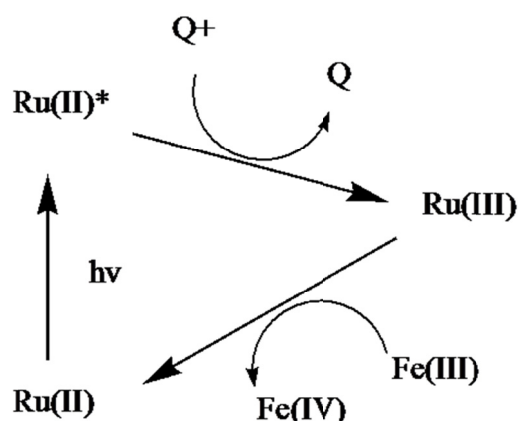
Figure 1.3. (A) Three-dimensional fold of gsNOS: the two peptide chains are shown in green and teal, with the heme highlighted in red. Note the striking similarity to the folds of bsNOS and iNOS (**Figure 1.2**). (B) An expanded view of the heme-thiolate active site of gsNOS, showing the axial cysteine ligand and a conserved tryptophan residue that hydrogen bonds to the thiolate and stacks with the porphyrin rings (PDB file 2FLQ).

1.4 An Interest in Heme-Thiolates

The Gray group has had a long-standing interest in heme-thiolate systems, specifically high-valent iron-oxo species long believed (and only recently proven) to be the active hydroxylating species in cytochromes P450. Our work on high-valent iron hemes actually began in the mid to late 1990s. The group had developed a technique called flash/quench, a general scheme of which is shown in **Scheme 1.4**.⁴⁵ In this process, a photosensitizer such as ruthenium(II) tris(2,2'-bipyridine (or bpy)) is excited by illumination with visible light (into its metal to ligand charge transfer band) creating an excited state with a lifetime of more than 600 μs (the flash). Interestingly, this excited state has a significant driving force to either gain or lose an electron, about 0.8 V.⁴⁶ In the presence of another reactant, such as ruthenium(III) hexaammine, the excited state reacts (is quenched) to form Ru(II) hexaammine and Ru(III)(bpy)₃. This Ru(III)(bpy)₃ species is an incredibly potent oxidant, with a driving force of nearly 1.3 V (in aqueous solution). Not only does flash/quench provide a more potent reactant, but often the further separation of charges produces a longer lifetime for the oxidizing species, allowing more time for the desired reaction to occur. (This same flash/quench scheme can be performed using a reductive quencher such as octacyano molybdate to produce the strong reductant Ru(I)(bpy)₃.)

Both reversible and irreversible quenchers can be used. In reversible systems, the quencher eventually reacts with either Ru(III) or another oxidized species to reform all of the original species in their resting oxidation states. For irreversible systems, once the quencher reacts with the excited photosensitizer it undergoes further chemistry,

preventing any back reactions; the system can never return to its resting state and with each laser pulse the reactants are consumed. Co(III) complexes are typical irreversible oxidative quenchers — once reduced, the ligands become labile and are replaced by water to form the hexaaquoCo(II) complex, which is much more difficult to oxidize again. The development of this technique has allowed the Gray group to study several interesting reactions that cannot occur on the timescale of the Ru(II)^* excited state.



Scheme 1.4. An oxidative flash/quench scheme, where Ru represents the photosensitizer Ru(bpy)_3 , Fe represents the iron within the heme cofactor, and Q is an oxidative quencher such as ruthenium hexaammine (so named because of its effect on the photosensitizer).

One such reaction viewed only using flash/quench involves the heme system microperoxidase-8 (MP8).⁴⁷ MP8 is a peptide containing only 8 amino acids, including two cysteines which form thioether links to a *c*-type heme. Remarkably, this tiny peptide and cofactor can still carry out peroxidation reactions. In an attempt to generate high-valent iron species in this histidine-ligated heme, researchers combined MP8, Ru(bpy)_3 and $\text{Ru(NH}_3)_6$ in buffered aqueous solution. Upon irradiation with a 10 ns pulse of 470 nm light from an Nd:YAG-pumped OPO (optical parametric oscillator), the Ru(II)(bpy)_3^* excited state forms. This then reacts with the $\text{Ru(NH}_3)_6$ on the nanosecond timescale to form Ru(III)(bpy)_3 . This Ru(III) species then oxidizes the iron atom of MP8

to form an oxidized, formally Fe(IV) species. Both Compound II (ferryl) and Compound I (ferryl + porphyrin radical cation) were observed. This process was repeated with the enzyme horse radish peroxidase (HRP).⁴⁸ With this system, and irreversible oxidative quencher was needed in order to afford enough time to transfer an electron from the heme center to the Ru(III) species. The characterization of these species furthered our understanding of their catalytic cycle.

The group then wished to extend this process to generate high-valent iron species in more complex systems, particularly cytochromes P450. Unfortunately, this afforded no detectable reaction. In fact, the use of irreversible quenchers led only to the degradation of their protein systems. The highly oxidized Ru(III) will find something to react with, even if it cannot perform the desired reaction with the iron, effectively leading to oxidative destruction of the protein.

In an effort to observe these elusive high-valent species in a cyt. P450, the group then began developing what later came to be called “wires”. Wires are modified photosensitizers, similar to the traditional Ru(bpy)₃ but with an additional component.⁴⁹ In examining the crystal structures of HRP and cyt. P450s, it became clear that while the heme of HRP was exposed to solvent (and therefore solution) on one edge, the heme of cyt. P450 was completely buried by the protein backbone. Researchers needed a way to promote interaction between the photosensitizer and the active site. The second component of these wires addressed this issue of coupling to the protein by attaching a tail group to the photosensitizer head (**Figure 1.4**). The tail group typically resembled the substrate of the particular cyt. P450 under study, bringing the Ru moiety closer to the heme, in effect, wiring the two together.

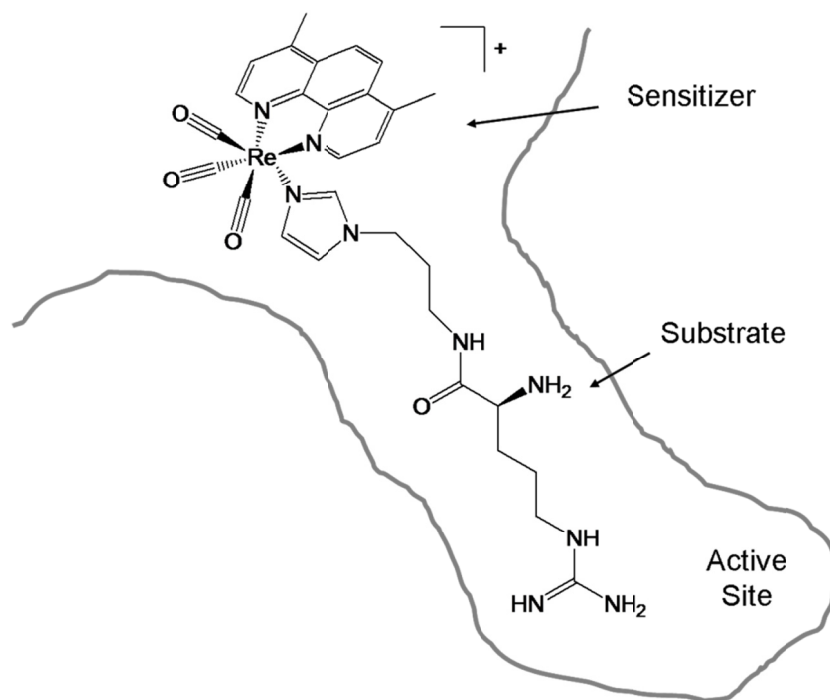


Figure 1.4. Schematic representation of a rhenium wire linked to an arginine-like tail binding within the NOS substrate binding pocket.⁴⁹

While these wires were never successfully used to generate high-valent iron, they were shown to photochemically reduce the iron centers of both cyt. P450 BM3 and iNOS.⁵⁰ Several systems were developed for each enzyme and their binding thoroughly characterized, but while forward electron transfer to the heme was observed in high yield, catalysis was blocked by the presence of the wire in the substrate binding pocket. This project did, however, reveal several interesting aspects of the nature in which cyt. P450s bind their substrates.⁵¹ The presence of a second heavy metal assisted in crystallization efforts and allowed researchers to observe significant conformational changes not before observed. The Gray group has also characterized both of these enzyme electrochemically, but again, without production of high-valent species.⁵²⁻⁵³ The goal of generating these species is ultimately to understand their catalytic cycles more completely, and in NOS in

particular there remain many questions about the exact mechanism by which NO is produced.

1.5 Tools of the Bioinorganic Chemist

The ultimate goal of the work presented herein is to further our understanding of the catalytic cycle of nitric oxide synthases in particular and heme-thiolates in general. There are many techniques for characterizing a metalloenzyme and its mechanism, even beyond those previously used by our group. One technique of great use to the Gray group is electronic absorption spectroscopy (UV-vis). This technique is particularly useful in the case of heme enzymes due to their characteristic absorption bands in the visible region of the electromagnetic spectrum. Both the Soret band and the Q bands are sensitive to oxidation state and ligation of the iron.¹⁸ Several examples of various common oxidation states with typical axial ligation (the sixth position, other than the four coordinating porphyrin nitrogens and the axial cysteine ligands) are shown in **Figure 1.5**. Shifts in Soret position (the intense band near 400 nm) are significant enough to allow a researcher to identify oxidation or ligation state often by simple UV-vis characterization.

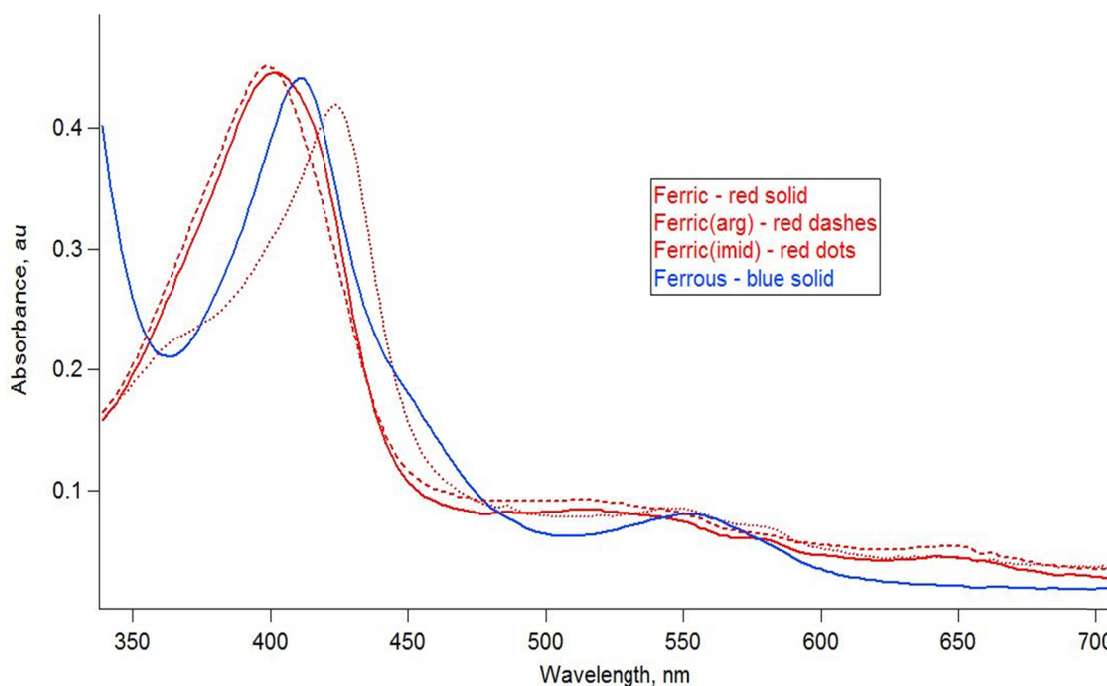


Figure 1.5. UV-visible absorption spectra of several oxidation and ligation states of wild type gsNOS. Note the shift in Soret band from 400 to 410 upon reduction of ferric (red solid) to ferrous (blue) and a shift to 426 nm upon introduction of the ligand imidazole (imid) to the ferric heme (red dots).

Several other techniques have been developed to allow chemists to gather extensive information about metalloenzymes. One such technique, electron paramagnetic spectroscopy (EPR), aids in assignment of spin state. This is incredibly important in these heme-thiolate systems where the energy difference between high-spin and low-spin iron complexes is small and thus both states are possible.²⁹ It has been demonstrated in other non-heme iron systems that spin state has a huge effect on the reactivity of the complex, altering reactivity by six orders of magnitude.⁵⁴ Definitive assignment of the spin state is often incredibly important. Another technique, electrochemistry (Echem), also yields valuable information on an enzyme under investigation. Echem informs about the reduction potential of a metal complex and often on its kinetics and further reactivity.⁵⁵ Several different electrochemical techniques can be employed, such as redox titrations to

determine an exact reduction potential and cyclic voltammetry to learn about the kinetics of such systems. These are just a few of the techniques available to a modern bioinorganic chemist, and such techniques will be introduced and explained further as they are used in the following work.

The final, but possibly most important, tool available to enzymologists (and chemists) today is site-directed mutagenesis. This process (awarded the Nobel Prize in Chemistry in 1993) allows researchers to select particular amino acids within a protein's sequence and change them into another amino acid, through creative use of primers and the polymerase chain reaction (PCR). Proteins can now be investigated and modified on the atomic level.

1.6 Conclusion

These techniques were used to investigate the nitric oxide synthase from *Geobacillus stearothermophilus* and its reactivity, with the goal of furthering our general understanding of NOS enzymes and their mechanism of NO production. This particular system provides stability not present in other NOSs and can be expressed in high yields in *Escherichia coli* in the lab (8 mg/L). Investigations were made into the nature of the heme active site and its reactivity.

This was done using two sets of mutant enzymes. The first set of mutants was designed to perturb the hydrogen bonding to the axial thiolate ligand. The native Trp was replaced in turn with His (which can still H-bond but cannot π -stack with the porphyrin), Phe (which can π -stack but not H-bond) and Tyr (again it can π -stack but not H-bond, but the hydroxylate group greatly alters its electronics). These mutants were analyzed using

various bioinorganic techniques, compared with the wild type, and used to learn about the tuning of the heme cofactor for the exact reactivity of NOSs.

The second set of mutants was made to investigate rates of NO release from the enzyme, once produced during catalysis. Different cellular functions of NO would require different rates of NO production and release.² It has been observed that two particular positions may be involved in gating NO release (positions 134 and 223 in gsNOS). Mutations were made to vary the bulk of side chains at these positions, and their rates of NO release and interactions with the diatomic mimic carbon monoxide (CO) were measured in detail. This thesis covers the work done investigating these two sets of mutants and the information gleaned from these experiments.

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