Chapter 7

Conclusions and Future Work

7.1 Roles of the Hydrogen-Bond Donating Groups in Tuning the Axial Thiolate

The thermodynamics of wild type and three mutants of gsNOS were characterized by various methods. Data from circular dichroism spectroscopy shows that mutations at position 70 do not decrease the overall stability of the protein fold. The evidence from multiple techniques is clear, however, that these mutations significantly affect the electronics of the heme center. It was shown using binding assays, generation of the ferrous-CO species, and redox titrations that the σ-donating abilities of the thiolate are increased after removal of the hydrogen bonding group in the Trp. Both chemical redox titrations and instability of ferrous-CO complexes of the two mutants lacking this key hydrogen bond (W70F and W70Y) suggest that they have more negative reduction potentials than the two mutants with this hydrogen bond (wild type and W70H). Evans Methods NMR was used to confirm the results of UV-visible spectroscopy which suggest that removal of this hydrogen bond shifts the heme center toward the high-spin state due to strengthening of the Fe-S bond, as seen in the binding assays.

It can be concluded that this universally-conserved tryptophan residue serves several roles, but positioning of the heme within the protein (as has been suggested for cyt. P450s) is not one of them. In order to produce NO the electronics of the heme center must be tuned in such as way as to stabilize high-valent iron species for the oxidation of substrate. During catalysis, the site must also be tuned not only to support the six-coordinate ferrous-oxy complex, but promote release of NO· from the heme in the end. If the 3/2+ reduction potential of the site is too negative, the ferrous-oxy may be unstable or the high-valent iron species too stable to perform the desired reactivity. If too negative, release of NO will be disfavored and decrease the rate of release to undesirable levels.

Stopped-flow coupled with UV-visible spectroscopy was employed to characterize wild type gsNOS and these three new mutant enzymes. It was shown that their autoxidation rates correlate with reduction potential data discussed in Chapter 3. The histidine mutant has an elevated reduction potential and the slowest autoxidation rate relative to the other three. The wild type is more negative by approximately 20 mV with a potential of -362 mV vs. NHE. This reduction potential is similar to that of mammalian inducible NOS, but these two are then more negative than other NOS enzymes by 100 mV. The reason for this behavior in gsNOS is unknown, but in iNOS the presence of the substrate sterically excludes a water molecule that coordinates the heme and this binding event shifts the reduction potential into the normal range for NOSs. The two mutants lacking this conserved hydrogen bond, W70F and W70Y, have significantly more negative potentials and were found to have very fast rate constants for autoxidation, consistent with more negative potentials.

The production of NO_x species of all four enzymes was characterized by the Griess Assay. The wild type produces nitrogen oxide species at a rate similar to other NOSs. The W70H mutant has an elevated rate of NO_x release/formation. The two mutants without this hydrogen bond have significantly decreased rates of NO_x production. Clearly this hydrogen bond plays a role in controlling the rate of NO release from the enzyme or the speed with which it is formed (as all four should react sufficiently rapidly with hydrogen peroxide for reduction not to be a factor in this assay).

Finally, stopped-flow was once again employed in order to determine if the Griess Assay was indeed detecting $NO\cdot$ or rather NO^- which are indistinguishable by that method. The ferric-NO complex, the immediate precursor to the nitric oxide product, was

observed for three of the four enzymes. Interestingly, this could not be observed for the W70H mutant. This mutant most likely releases NO^-.

The conserved proximal hydrogen-bond donating group found near the axial thiolate ligand in all nitric oxide synthases plays a key role in tuning the electronics of the active site. This is a uniquely long hydrogen bonding interaction between this tryptophan and the thiolate, at just 3.7 Å. Without this interaction, the enzyme is still capable of producing NO, as found for both the W70F and W70Y mutants by single turnover experiments. Their reduction potentials, however, are incredibly negative and most likely fall far below the biologically relevant window. The replacement of this tryptophan with a histidine results in an enzyme with a more elevated potential, however it cannot release NO radical. The histidine residue, lacking the aryl ring, most likely cannot π-stack with the porphyrin ring, giving it more flexibility. This may allow it to move closer to the thiolate to improve this hydrogen bonding interaction. If this interaction is too strong, NO^- is released.

In the second turnover of the catalytic cycle, an electron from the heme center must be shuttled back into the tetrahydrobiopterin cofactor to re-reduce it. The potentials of both the heme and the pterin must be tuned perfectly to allow forward electron transfer into the ferrous-oxy complex followed by back electron transfer into the pterin. This back electron transfer allows release of $NO\cdot$ and not NO^-. If the potential of the heme center is too high, this back electron transfer cannot occur, preventing $NO\cdot$ release. Thus, the hydrogen bonding interaction is necessary for tuning the reduction potential high enough for the reduction of the heme by a reductase domain/enzyme. However, when too strong,

the potential is tuned too high to send an electron back into the pterin after catalysis, which is necessary for formation of the product NO.

7.2 Gating of Diatomics in Nitric Oxide Synthase

First, CO is a valuable diatomic mimic for the more reactive dioxygen and nitric oxide, however with a caveat. One must remember the conditions under which experiments are performed. For CO photolysis, systems are under saturating conditions with large excesses of carbon monoxide. This is a good system for comparison with oxygen binding to hemoglobin in the lungs. Nitric oxide formation, on the contrary, involves the production of a single molecule of NO per protein, far from saturating conditions. Further, NO reacts rapidly in aerated aqueous solution, further preventing its buildup and keeping the system from reaching equilibrium. Also, the driving force ultimately behind each of these processes involves formation or cleavage of two distinct bonds. CO is very similar to NO, but on a fundamental level an Fe-N bond is not an Fe-C bond. While experiments with CO provide a wealth of information about the overall kinetics model for reactivity with diatomics, for an NO release rate one must perform the single turnover experiments.

With these experiments, we have confirmed that the isoleucine residue at position 223 does gate NO release in gsNOS, slowing the decay of the ferric-NO species. We have also demonstrated that position 134, occupied by a histidine in wild type gsNOS, also gates NO release, with smaller residues at this position corresponding to faster release rates. Together, these two positions can account for the majority of the differences in rate between any two NOS enzymes.

Further, we have used a new, more accurate five-state model to fit our data showing rapid equilibrium between the bound and unbound NO, and another unidentified species. This species has been previously observed in a mammalian enzyme by another group. It was called Compound I. The Compound I in cytochromes P450 has a much more blue-shifted Soret band with a maximum near 365 nm and it reacts much more quickly. It is of note that this species with absorbance at 420 nm has now been observed in both turnovers. This species could possibly be some form of the P420 species in NOS, which has either lost thiolate ligation or the thiolate has become protonated, becoming a neutral thiol ligand. Unfortunately, its exact identity remains uncertain.

7.3 Future Directions

Several experiments remain in order to fully characterize the set of mutants probing the role of the hydrogen bonding to the axial thiolate. First, redox titrations must be conducted with a different chemical titrant. The 3/2+ couple of $Ru(acac)_3$ is too positive to determine the potential of the W70F and W70Y mutants. Another reagent, such as benzyl viologen with a reduction potential of -374 mV vs. NHE, might prove more appropriate for observing the equilibrium between oxidized and reduced species. Further, these titrations should be carried in the presence of the substrate arginine as well, in order to determine if the presence of substrate shifts the potential as was seen with mammalian inducible NOS.

The full effect of mutating this hydrogen bond donor cannot be understood until structural data is collected. Crystallography would answer questions about the distance of each residue from the thiolate and from the porphyrin ring, as well as the positioning of

the sulfur atom with respect to the iron for each sample. It would also reveal any other unexpected effects on the fold of the protein. Samples have been sent to the lab of Brian Crane at Cornell, an expert in NOS crystallography. This family of enzymes, however, is notoriously difficult and slow to crystallize. Efforts to obtain these three-dimensional structures are underway.

Work on protein film voltammetry with gsNOS is still ongoing in the lab of Michael Hill at Occidental College. In addition to measuring reduction potentials, the interaction of each sample with the axial water ligand will be investigated by studying the scan rate dependence. The iron centers will also be probed in the presence of arginine, which should shift all potentials and remove any interaction with water at the heme. These experiments are expected to be completed within the next couple of months.

Finally, electron transfer pathways in NOS should be studied using more traditional Gray group laser-induced flash/quench techniques. Attempts have been made at connecting a Ru-trisdiimine photosensitizer to the enzyme in order to induce ET to/from the heme (see Appendix II). The iodoacetamidophenanthroline ligand should be used to tether $Ru(bpy)_2$ to the enzyme at position 115. Initial studies show quenching of the Ru excited state and formation of Ru(III), but no oxidation of the heme despite rapid loss of the Ru(III). These results suggest that possibly another residue in the enzyme is being oxidized. gsNOS has more than a dozen oxidizable Trp and Tyr residues. Mutations may be required in order to promote oxidation of the heme center rather than another residue. Further studies should be able to produce high-valent heme species in this enzyme. The characterization of any high-valent species in NOS would be of incredible interest to the field, as the active oxidants are still unknown.