Engineering Myoglobin into Both a Structural and Functional Model of Nitric Oxide Reductase

Natasha H. Yeung Final Seminar November 24, 2008

Nitric oxide reductase (NOR) is the only metalloenzyme in the denitrification pathway for which there is no crystal or solution structure. NOR catalyzes the two electron reduction of NO to N_2O. From sequence alignments and homology modeling, NORs are predicted to be structurally homologous to subunit I of HCOs (heme-copper oxidases), enzymes that catalyze the reduction of O_2 to water. However, the copper site (Cu_B) in HCO is replaced with a non-heme iron site (Fe_B) in NOR (Figure 1).[1,2] There are several conserved glutamates in NOR that are not found in HCO, but only one to two glutamates are predicted to be in vicinity of the Fe_B site.[3,4] A glutamate is proposed to play a role in iron binding to the Fe_B site and/or catalysis. In addition to their structural similarities, NORs and HCOs also have cross-reactivity. NOR is capable of O_2 reduction and some members of the HCO superfamily are capable of NO reduction.[5] Therefore, an important question is whether the identity of the metal at the Cu_B site in HCO and the Fe_B site in NOR, help tune activity in homologous protein metal binding sites.

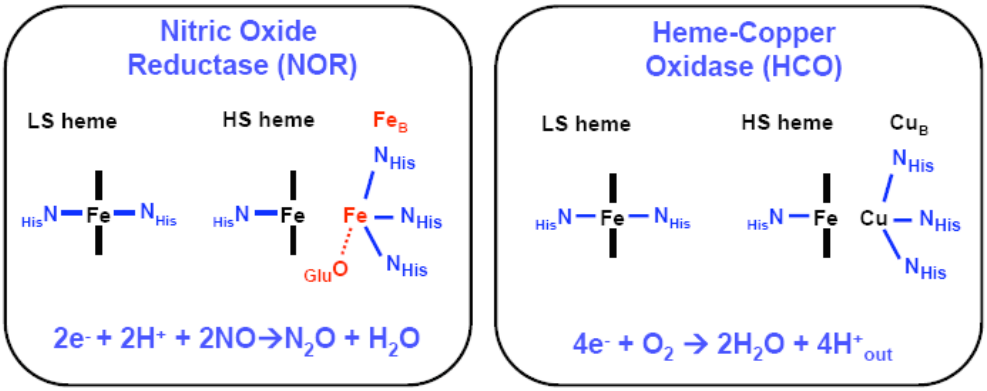

Figure 1: Proposed active site structure of bacterial nitric oxide reductase (NOR) and the predicted role of the conserved glutamate in NOR as a ligand to the Fe_B site (left) and comparison to the active site structure of heme-copper oxidase (HCO) (right).

Synthetic protein models are often either structural or functional models, and native NOR is difficult to study due to its size, complexity, and the presence of multiple chromophores. Biosynthetic protein models which act as a bridge between synthetic systems and studies of native proteins are ideally suited to metalloprotein modeling. The biosynthetic approach involves the engineering of a native protein active site into a smaller scaffold protein, chosen for its easy to purify, easy to characterize, and well characterized nature. As well, different metals can be easily introduced into many biosynthetic protein models, allowing for the study of the effect each metal has on protein structure and function. This task is not easily achievable in native proteins, oftentimes leading to inactive and/or unstable protein.

Our group has engineered a Cu_B site into the scaffold protein myoglobin (Cu_BMb, swMb L29H, F43H, H64; swMb is sperm whale myoglobin).[6] Given the structural similarities between HCOs and NORs, a closer structural model of the NOR active site was constructed by introducing a glutamate ligand near the heme of Cu_BMb, called Fe_BMb (swMb L29H, F43H, H64, V68E), to mimic the conserved glutamate(s) in NOR.

Crystallographic and/or spectroscopic studies show that Fe_BMb can bind Fe or Cu in its engineered Fe_B site. The crystal structure of Fe(II)-Fe_BMb (Figure 2) shows that the non-heme iron (Fe_B) is bound to all three histidines in the engineered metal binding site (2.24 Å to His29, 2.36 Å to His43, and 2.20 Å to His64), one oxygen atom of Glu68 (2.03 Å), and one water molecule (1.92 Å). An additional H-bond to the other O atom of Glu68 (2.73 Å) is likely formed.

From GC/MS and UV-vis studies, Fe_BMb was found to reduce NO to N_2O in the presence of Fe(II) or Cu(I) under one turnover conditions. However, NO activity is lower with Fe than with Cu bound to the same protein scaffold. In contrast, Cu_BMb, which does not contain a glutamate ligand near the heme, showed no evidence of either Fe binding or NO activity with Fe. These results support the vital role that the glutamate plays in retaining the iron in the Fe_B site.

The introduction of a glutamate in close proximity to the heme (i.e., Fe_BMb, $E_{1/2}$= -158 mV) appears to lower the reduction potential of the heme by >200 mV, when compared to Cu_BMb ($E_{1/2}$= +77 mV). The modulation of the heme potential by a nearby glutamate also mimics that of NOR, where the catalytic heme reduction potential was found to be much lower (>200 mV) than the surrounding cofactors.[7]

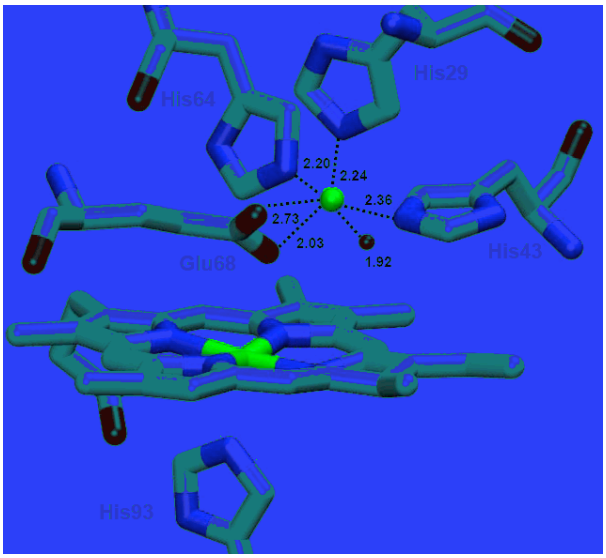

Figure 1: Crystal structure of Fe(II)-Fe_BMb (1.8 Å resolution, data collected at Fe absorption edge). Fe_B is represented by a green sphere and a water molecule by a red sphere. A heme propionate group has been omitted for clarity.

Since HCOs and NORs have cross-reactivity, our Cu_B site model Cu_BMb, was investigated for NO activity with Cu (as there was no reaction with Fe, *vide supra*). Copper-bound Cu_BMb catalytically reduces NO to N_2O at a rate of ~2 mol NO□mol $Cu_B Mb^{-1}$□min^{-1}, making Cu_BMb a functional model of NOR.[8]

To date, Fe_BMb is the first protein model of the Fe_B site of NOR and provides the first evidence, from an NOR model, that the identity of the metal in analogous non-heme metal binding sites of HCO (i.e., Cu_B) and NOR (i.e., Fe_B) can tune NO activity. In summary, Fe_BMb is both a structural and functional model of NOR, and Cu_BMb is a functional model of NOR, and as such, will serve as excellent models for mechanistic studies of NOR, enabling the study of the effect that Cu and Fe has on NO activity.

References

1. Dermastia, M.; Turk, T.; Hollocher, T.C. Nitric Oxide Reductase. Purification from Paracoccus denitrificans with Use of a Single Column and Some Characteristics. *J. Biol. Chem.* **1991**, *266*, 10899-10905.

2. Sakurai, N.; Sakurai, T. Genomic DNA Cloning of the Region Encoding Nitric Oxide Reductase in Paracoccus halodenitrificans and a Structure Model Relevant to Cytochrome Oxidase. *Biochem. Biophys. Res. Commun.* **1998**, *243*, 400-406.

3. Hendriks, J.; Gohlke, U.; Saraste, M. From NO to OO: Nitric Oxide and Dioxygen in Bacterial Respiration. *J. Bioenerg. Biomembr.* **1998**, *30*, 15-24.

4. Butland, G.; Spiro, S.; Watmough, N.J.; Richardson, D.J. Two Conserved Glutamates in the Bacterial Nitric Oxide Reductase are Essential for Activity but Not Assembly of the Enzyme. *J. Bacteriol.* **2001**, *183*, 189-199.

5. Wasser, I.M.; de Vries, S.; Moeenne-Loccoz, P.; Schroeder, I.; Karlin, K.D. Nitric Oxide in Biological Denitrification: Fe/Cu Metalloenzyme and Metal Complex NOx Redox Chemistry. *Chem. Rev.* **2002**, *102*, 1201-1234.

6. Sigman, J.A.; Kwok, B.C.; Lu, Y. From Myoglobin to Heme-Copper Oxidase: Design and Engineering of a CuB Center into Sperm Whale Myoglobin. *J. Am. Chem. Soc.* **2000**, *122*, 8192-8196.

7. Groenberg, K.L.C.; Roldan, M.D.; Prior, L.; Butland, G.; Cheesman, M.R.; Richardson, D.J.; Spiro, S.; Thomson, A.J.; Watmough, N.J. A low-redox potential heme in the dinuclear center of bacterial nitric oxide reductase: Implications for the evolution of energy-conserving heme-copper oxidases. *Biochemistry* **1999**, *38*, 13780-13786.

8. Zhao, X.; Yeung, N.; Russell, B.S.; Garner, D.K.; Lu, Y. Catalytic Reduction of NO to N2O by a Designed Heme Copper Center in Myoglobin: Implications for the Role of Metal Ions. *J. Am. Chem. Soc.* **2006**, *126*, 6766-6767.