Appendix II

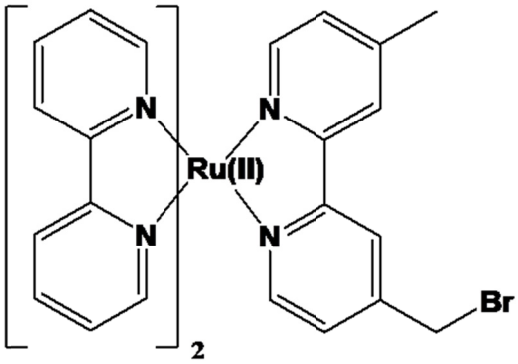
Labeling gsNOS with Ru-diimine Photosensitizers

AII.1 Introduction and Summary

The Gray group's interest in NOS focuses on the nature of the putative high-valent intermediates in the catalytic cycle. To study these, we have expressed a mutant with a single solvent-exposed cysteine residue and attached a ruthenium tris-diimine complex as a photosensitizer. We use the Ru complex and a laser-induced flash/quench scheme to pull electrons out of the active site to generate high-valent species, which we characterize by transient absorption spectroscopy.

In order to photochemically generate high-valent species of the heme center in nitric oxide synthase, mutant forms of the enzyme from *Geobacillus stearothermophilus* were expressed.* The enzyme contains more than a dozen surface-exposed histidine residues, so cysteine labeling is the preferred method for attaching the photosensitizer. There are four native cysteine residues in gsNOS at positions 76, 161, 227, and 269. Cys76 ligates the iron center and is necessary for enzymatic function. Cys161 is fully buried within the core of the protein and inaccessible to solution. Positions 227 and 269 were mutated to serine residues in order to prevent them from interfering with labeling reactions. First, a cysteine was installed close to the heme at position 84 (only 8 residues from the axial thiolate ligand). This position failed to label. Another position, K115, was mutated to a cysteine and this mutant was successfully labeled on two occasions (K115C/C227S/C269S). All of these plasmids can be found in the -20 alumni freezer in the box labeled Charlotte NOS.

The first molecule synthesized for labeling purposes was the photosensitizer shown below in **Scheme 1**. This molecule was attached to the mutant gsNOS protein on two occasions. Labeling conditions and synthetic details can be found below.



Scheme 1. The ruthenium photosensitizer used for labeling gsNOS.

AII.2 Materials and Methods

4-(bromomethyl)-4'-methyl-2,2'-bipyridine was synthesized according to published methods: Gould *et al*., *Inorg. Chem*. **1991**, *30*, 2942–2949, Strouse *et al*. *Inorg. Chem*. **1995**, *34*, 473–487, and Geren *et al.*, *Biochem*. **1991**, *30*, 9450–9457.

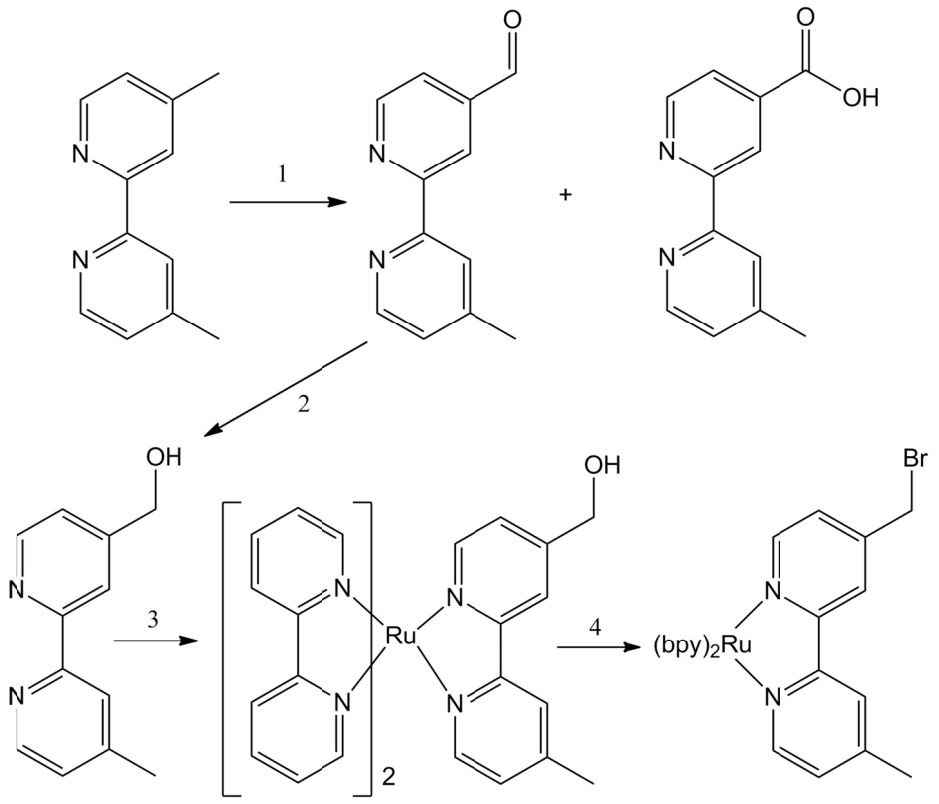
(**1**) 2g (0.011 mol) of 4,4'-dimethyl-2,2'-bipyridine were dissolved in 120 mL of dioxane (warm the mixture a little if it doesn't all dissolve). 2g of SeO_2 (0.018 mol) were added to this solution and it was refluxed for 24 hours. After cooling, the solution was filtered to remove the black precipitate. (The papers say to filter while hot to remove the black precipitate, let it cool, then again to collect a yellow precipitate. I found this unnecessary as no yellow precipitate ever crashed out.) The dioxane was removed by rotovapping and then the residue was redissolved in chloroform. The solution was again filtered to remove anything insoluble in chloroform. The chloroform was removed and the residue was redissolved in 200 mL of ethyl acetate. The ethyl acetate solution was extracted with 1 x 25 mL of 0.1 M $NaCO_3$ (aqueous) to remove the carboxylic acid product (see **Scheme 2**) which was discarded. The ethyl acetate solution was again extracted, this time with 3 x 30 mL of 0.2 M $NaHSO_3$ (aqueous sodium bisulfate solution). It turned pink. The pink aqueous fractions were then combined and extracted

with 4 x 25 mL of dichloromethane. The pink color remains in the aqueous fraction. The DCM is then rotovapped to give pure product. Yield, 20%. For NMR characterization see Strouse et al.

(**2**) 0.249 g of the product from (**1**) was dissolved in 15 mL of methanol. This solution was submerged in an ice bath. 6 mL of a solution of 0.2 M NaOH was prepared, and 55 mg of sodium borohydride ($NaBH_4$) was added to the NaOH solution. After ample time for cooling of the methanol, the borohydride was added dropwise to it. This mixture was allowed to stir on ice for a few minutes before removal of the ice bath and stirring for another hour at room temperature. The methanol was removed leaving a suspension of white solid in the aqueous fraction of the reaction. To this 6 mL of saturated Na_2CO_3 aqueous solution was added. This aqueous mixture was then extracted 4 times with 15 mL of chloroform. The chloroform was dried using $MgSO_4$ and then filtered and rotovapped to yield a white solid in > 90% yield.

(**3**) 193.6 mg (0.40 mmol) of cis-dichloroRu$(bpy)_2$ and 100 mg (0.50 mmol) 4-hydroxymethyl-4'-methylbipyridine (product of (**2**)) were added to a round bottom flask. This was dissolved in 100 mL of water and refluxed for 1 hour. It was then cooled, filtered, the liquid collected, and a solution of saturated NH_4PF_6 (aq.) was used to crash out the product. This was filtered and the product dried on a vacuum line.

(**4**) Once dry, the product from (**3**) was dissolved in 10 mL HBr and 1 mL H_2SO_4 and refluxed for approximately 5–6 hours. It was cooled and the product was precipitated using saturated NH_4PF_6 (aq.) again to precipitate the product. This was filtered and washed with ether and dried under vacuum to yield pure product.



Scheme 2. Synthesis of bromo-bpyRu(bpy)$_2$ photosensitizer.

This complex, the bromo-bpyRu(bpy)$_2$ photosensitizer, was used to label K115C/C227S/C269S gsNOS. Protein from 6L of growth was concentrated immediately after purification by gel filtration column (see Chapter 3). The entirety of clean gsNOS fractions was concentrated to under 10 mL in Tris buffer and desalted into 50 mM sodium phosphate buffer pH 8.0 (and 22 mL of roughly 10 μM NOS was collected). 45 mg of the labeling complex was dissolved in 3 mL of DMSO and then 5 mL of water was added to this, making a stock solution of approximately 6.7 mM. 0.44 mL of this Ru-containing solution was added to the 10 mL of protein along TCEP to a final concentration of 1 mM (22 L in μ22 mL). This solution was shaken in the cold room in the dark overnight (15 hours). A desalting column was used to remove the excess Ru complex. The solution was then loaded onto a HiTrap Q column. Buffer A = 50 mM

NaPi at pH 8.0, buffer B = 50 mM NaPi, pH 8.0 and 500 mM NaCl. A gradient was run from 0–70% B over 60 minutes at a rate of 2.5 mL/min (150 mLs or 30 column volumes). Elution was monitored at 280 nm (protein side chains), 400 nm (Soret band), and 460 nm (predominantly the Ru complex). Two large bands and one smaller shoulder came off the column, in that order, between 10 and 50% B. The mass spec of the first fraction corresponds to the mass of the protein (43863.6, calculated) plus the Ru complex (44460.8, calculated).

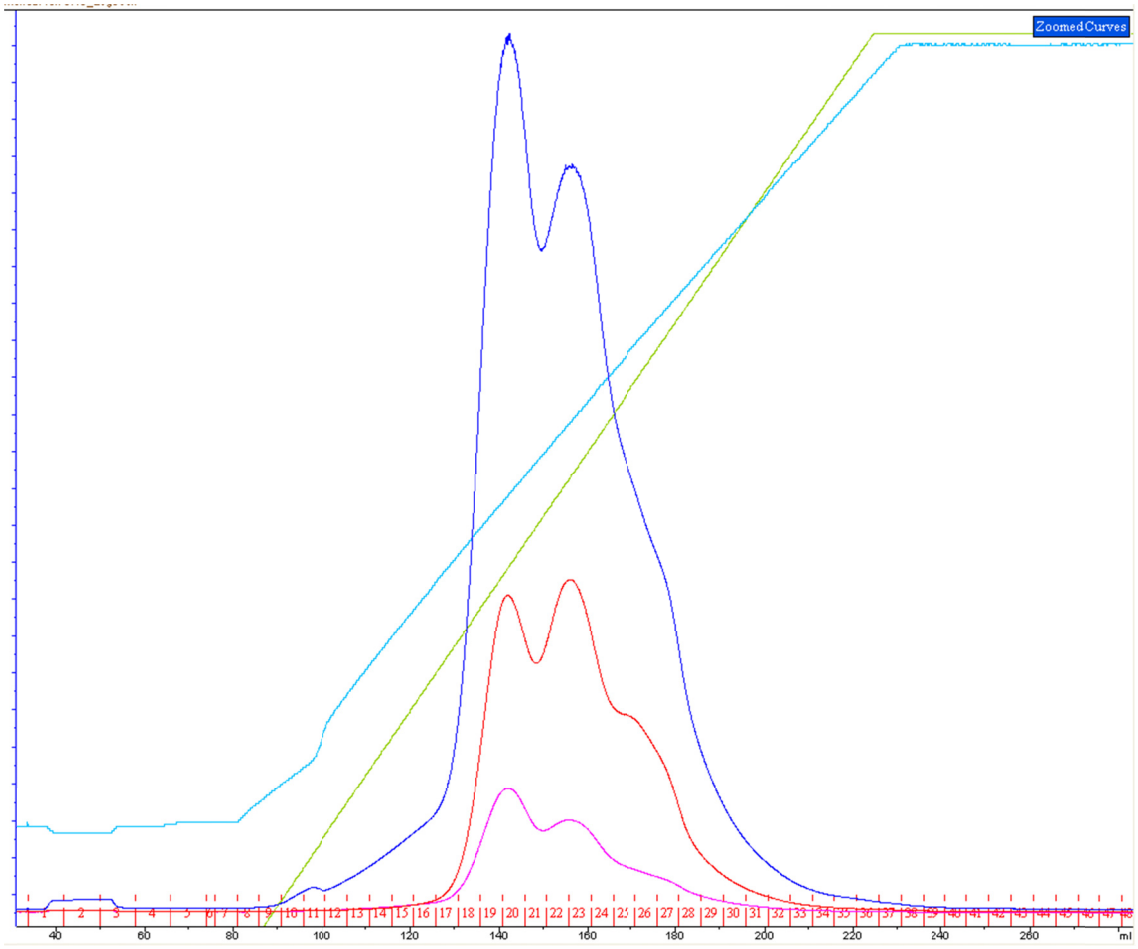


Figure 1. FPLC trace for labeled protein, the labeled fraction elutes first followed by unlabeled and another shoulder (mass corresponds to unlabeled as well).

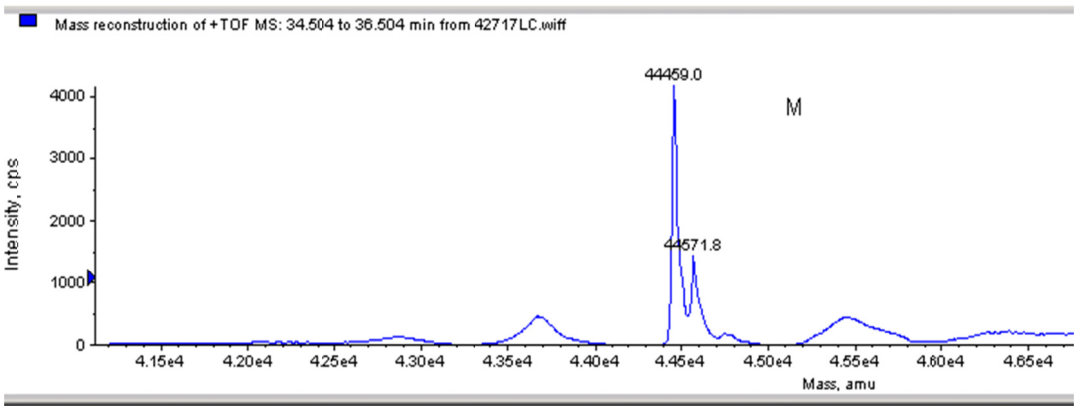


Figure 2. Mass spec of the first band to elute from Q column.

AII.3 Results

The presence of the label on the protein was confirmed by UV-vis and steady-state fluorescence spectroscopies.

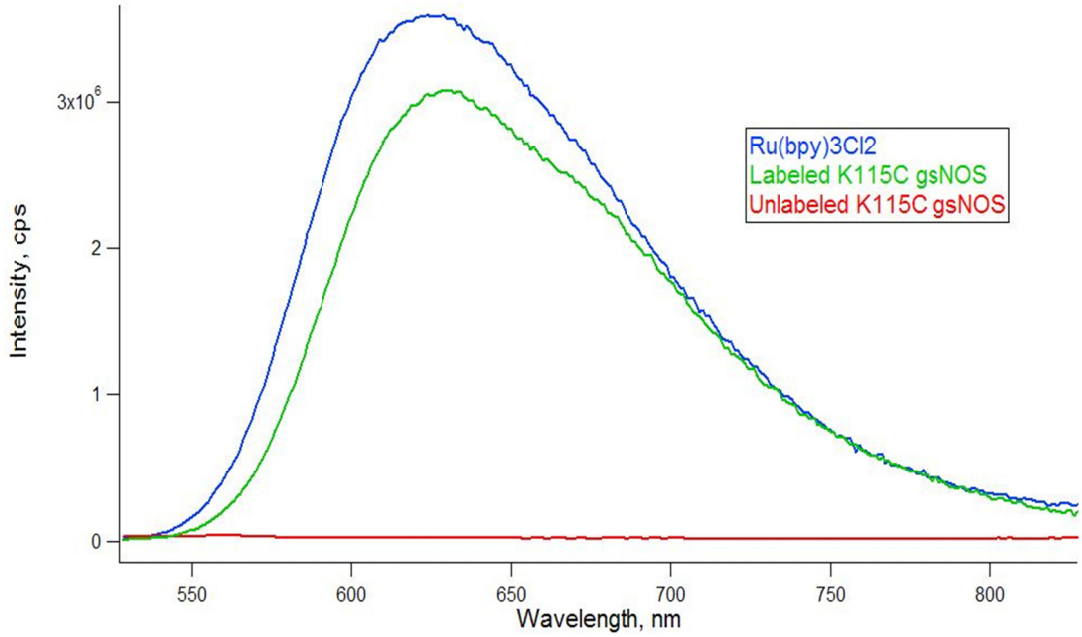


Figure 3. SSFL of labeled gsNOS in comparison with a comparable concentration of the model complex. A small amount of quenching is observed due to fluorescence resonant energy transfer to the heme in the enzyme.

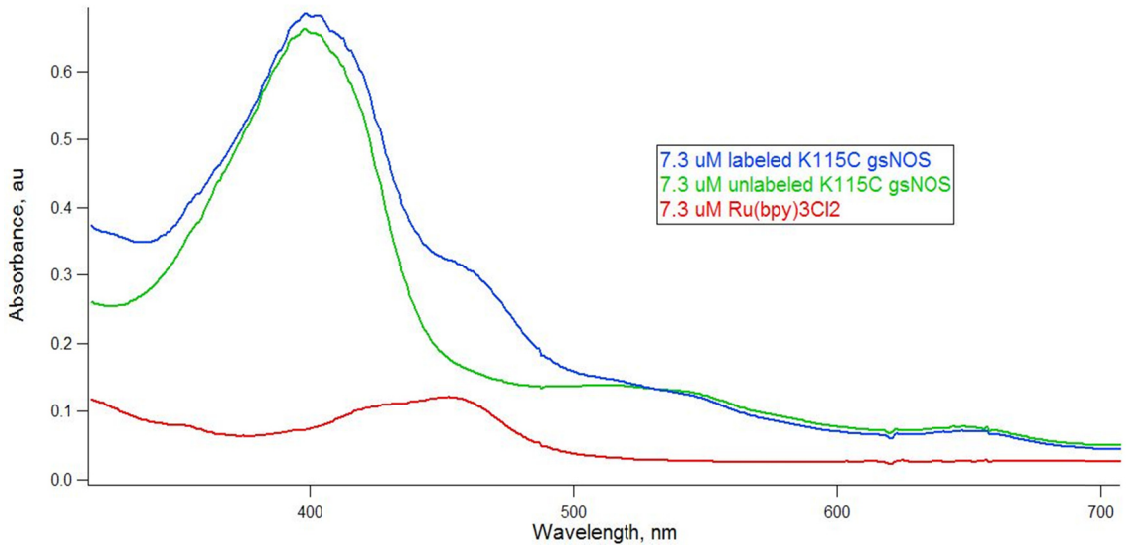


Figure 4. UV-vis spectra of model complex, unlabeled protein and the labeled protein.

The labeled NOS was then used in laser studies, with Ru-hexaammine as an oxidative quencher and $Ru(bpy)_3$ as a model complex for comparison.

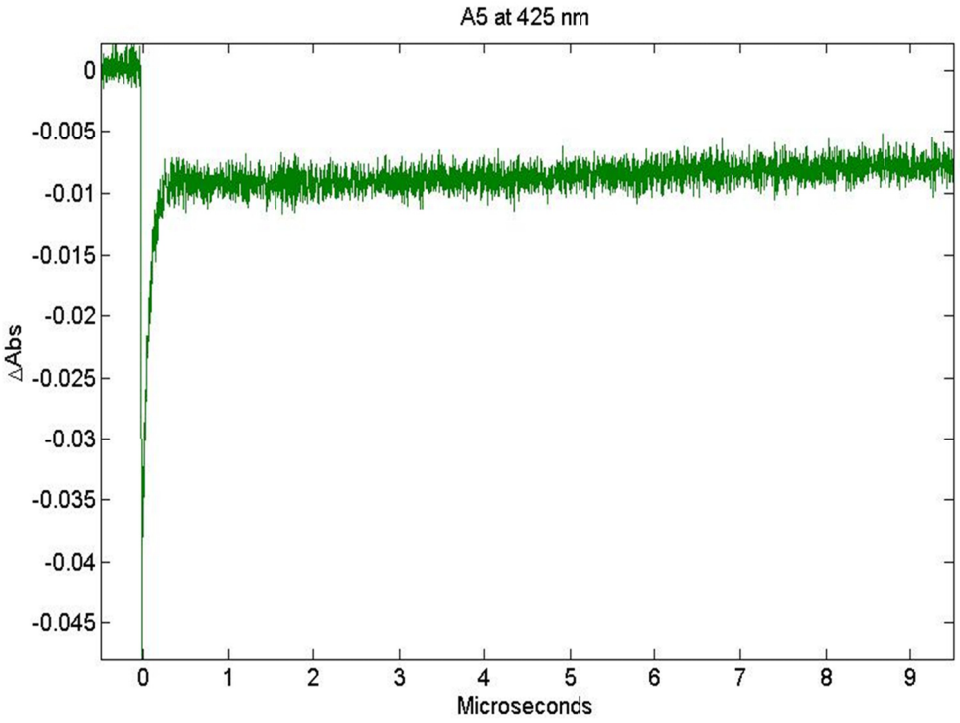


Figure 5. 8 µM $Ru(bpy)_3^{2+}$ + 5 mM $Ru(NH_3)_6^{3+}$ at 425 nm showing formation of Ru(III).

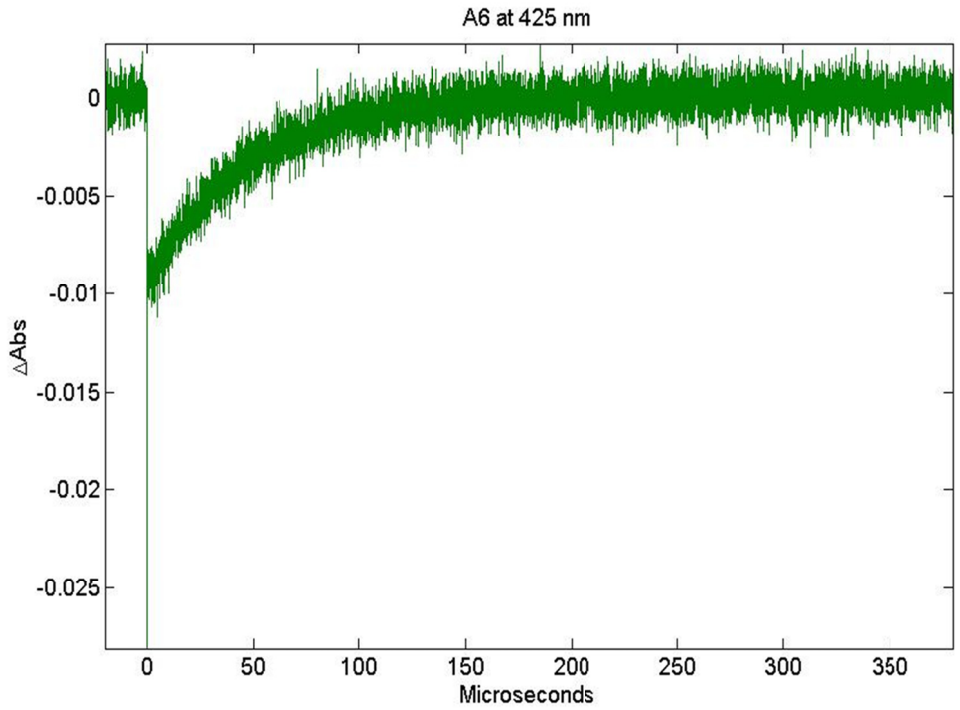


Figure 6. 8 µM $Ru(bpy)_3^{2+}$ + 5 mM $Ru(NH_3)_6^{3+}$ at 425 nm showing decay of Ru(III).

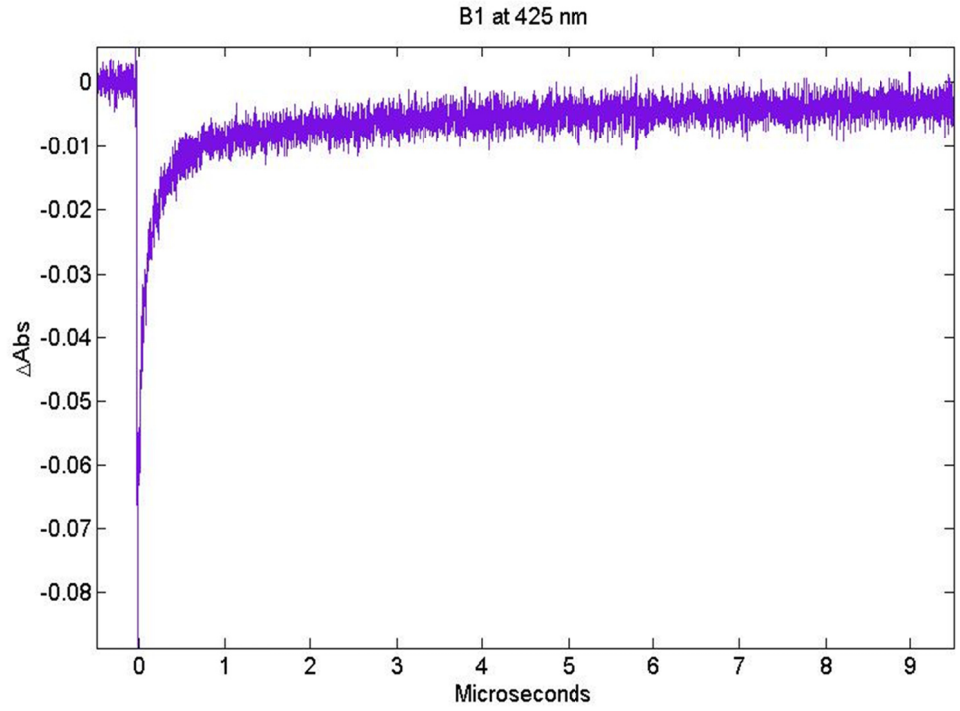


Figure 7. 8 µM labeled gsNOS + 5 mM $Ru(NH_3)_6^{3+}$ at 425 nm showing formation of Ru(III) again.

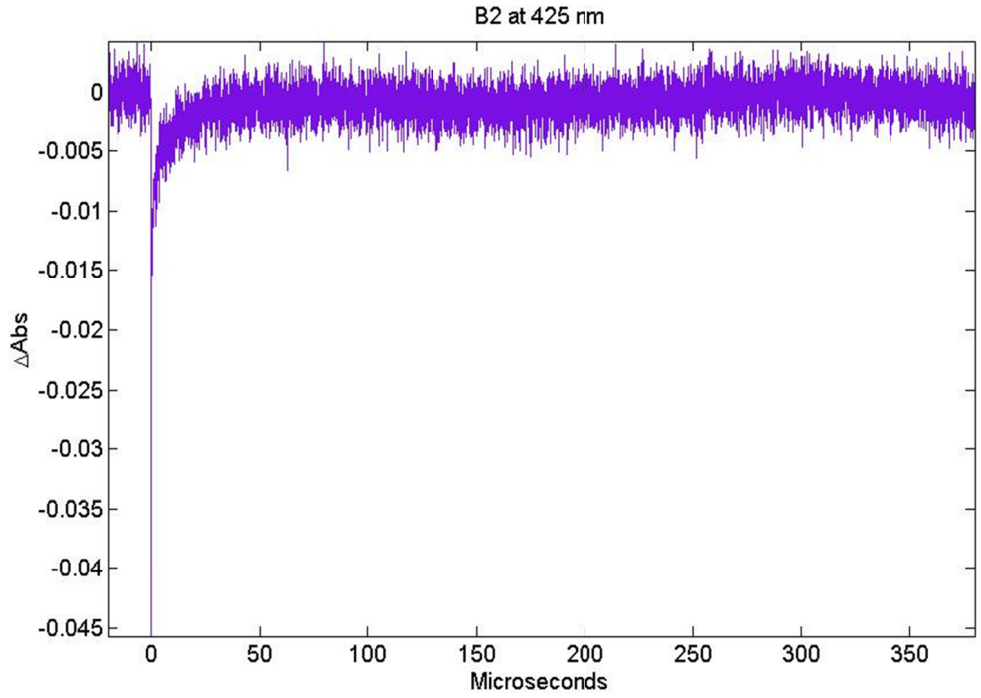


Figure 8. 8 μM labeled NOS + 5 mM $Ru(NH_3)_6^{3+}$ at 425 nm showing more rapid decay of Ru(III) in the presence of protein.

Unfortunately, no changes were observed in the Soret region, however, the Ru^{2+} signal recovers too quickly. Most likely, the strongly oxidizing Ru^{3+} is pulling an electron from another source, perhaps a tryptophan or tyrosine residue. A model was made (thanks to Kyle Lancaster) of the labeled protein, shown in **Figure 9**.

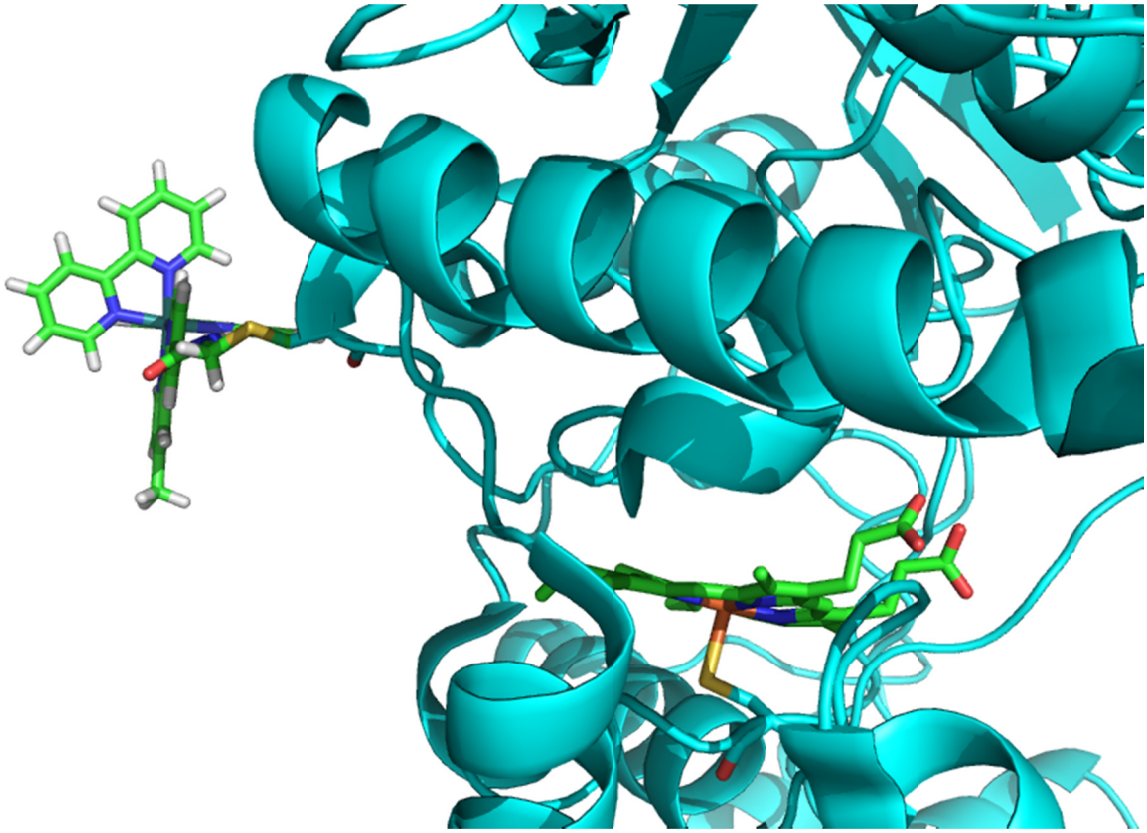


Figure 9. Model of the labeled protein showing proximity of the Ru to the heme.

Upon closer examination of the protein, we find 28 Trp and Tyr residues, including a tightly packed cluster, shown in **Figure 10**, including a patch that seems particularly close and easy to oxidize that contains a cluster of three of these residues (**Figure 11**). On its way to the heme, the hole may get side-tracked by these easily oxidized amino acids.

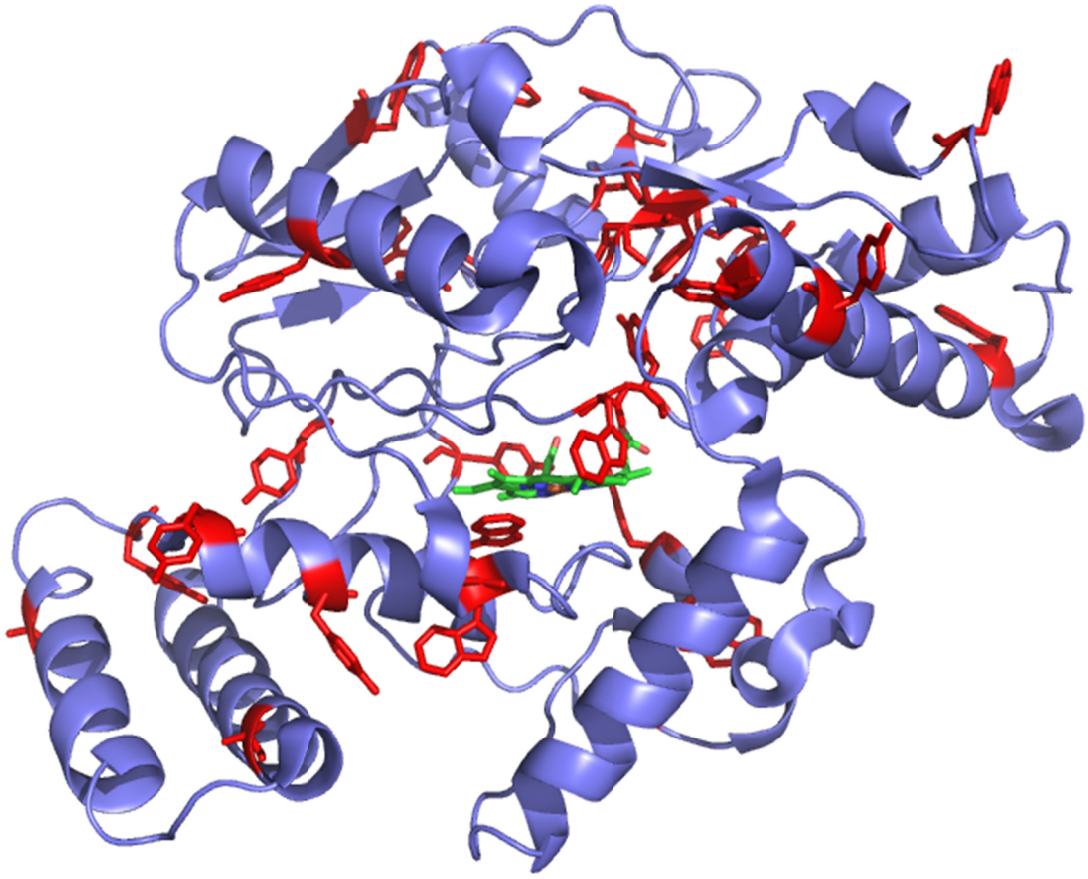


Figure 10. Wild type gsNOS showing all 28 Trp and Tyr residues in red.

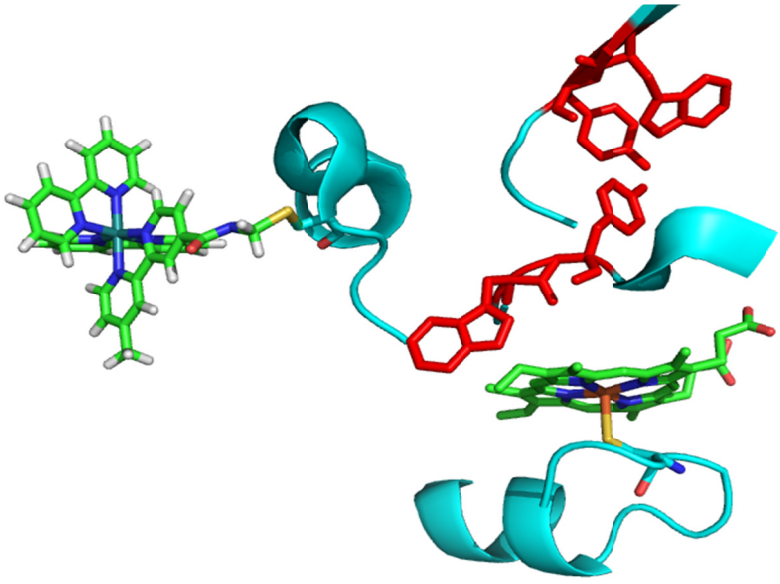


Figure 11. Model of labeled protein showing the location of several Trp and Tyr residues immediately between the label and the heme.

AII.4 Future Directions

This project was abandoned due to poor reproducibility of the labeling reaction and the lack of oxidation of the heme, as shown in the laser studies. We now know that reproducibility of the labeling reaction can be avoided by using an iodoacetamido-phenanthroline to label rather than an aryl bromide. See Ener *et al.*, *PNAS*, **2010**, *107*, 18783–18786. A note on synthesis of this new label: isolation was simplified by attaching aminophenanthroline to $Ru(bpy)_2Cl_2$ first (by a similar procedure used for step (**3**) above), and then mixing this complex with iodoacetic anhydride (1:1) in DCM and extracting this with an aqueous solution to remove iodoacetic acid. The product is light sensitive, so store it under foil. When this complex is used under the same labeling conditions as above or those published by Ener and coworkers, the labeling proved more reliable.

* Attempts were made to photochemically oxidize the heme with $Ru(bpy)_3^{2+}$ in the presence of both reversible and irreversible oxidative quenchers. In neither case were any oxidative products observed, therefore covalent attemchent was deemed necessary.