# AMINO ACID RADICALS IN RHENIUM-MODIFIED COPPER PROTEINS

Thesis by

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In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

2003

(Defended June 4, 2003)

## Acknowledgements

There are many people at Caltech whom I would like to thank for making my graduate experience personally and professionally enjoyable and memorable.

I first met Harry Gray in November 1995 when he came to the University of Arizona to give a seminar on electron transfer in ruthenium-modified proteins. At that time, I have to admit, the subject of his talk did not particularly interest me, even though I learned something and I actually remember the talk. It's amazing how Harry has that effect on people. Harry even frightened me when, as his host fiddled furiously through twenty light switches, he turned to the audience, scanned them, and said with a glowing grin: "I like to keep the lights on during my talk so that I can point out anyone who falls asleep." Not knowing Harry's jovial personality, I took him seriously. Six and a half years later, at a lecture in Copenhagen, as he started to present some of my results, Harry called out to me to get my attention. I was wide-awake, although Harry may have a different recollection. Either way, I figured that I had succeeded in the Gray group if Harry addressed me directly during a seminar. I would like to thank Harry for his generosity, for his support of me during my six years in graduate school, and for his incredible enthusiasm for science. Harry introduced me to the Big Picture, a guest at every group meeting, inorganic and organometallics seminar, Chem 153 lecture, and sundry meetings. He gave me the freedom to figure things out for myself and to go where my mind and my abilities would take me. This made the journey more difficult, but also more worthwhile when I made it to the end. Thank you, Harry.

I received all of my biological training in the labs of Doug Rees and Jack Richards and I thank them for their time, patience, and insight. I would also like to thank Mitchio Okumura and Nate Lewis (rounding out an All-Star committee) for their support and encouragement.

I would like to thank Jay Winkler who always provided me with his critical (and honest) assessment of the work that I did. Jay was rigorous in his demand for the details, while never losing sight of our friend, the Big Picture.

John Enemark gave a naïve freshman a chance to do chemical research in his lab nine years ago. I would like to thank him for getting my scientific career started and for supporting and encouraging me ever since.

The Gray group was a great place to be a graduate student, with outstanding colleagues and friends, whom I would like to thank: Malin Abrahamson, Gitrada Arjara, Wendy Belliston, Jesper Bendix, Steve Contakes, Alex Dunn, Cecelia Engman, Jasmin Faraone-Mennella, Mike Green, Corinna Hess, Kristine Jensen, Chip Kent, Judy Kim, Liz Krider, Brian Leigh, Mike Machczynski, John Magyar, Libby Mayo, Alex Meier, Kate Pletneva, Adrian Ponce, Jeff Rack, Karn Sorasaenee, Jason Telford, Akif Tezcan, Randy Villahermosa, Jeremy Weaver, Oliver Wenger, Jon Wilker, and certainly my classmates Xenia Amashukeli, Jenn Lee.

I would like to thank Pat Anderson, Sherry Feick, Kathleen Hand, Margot Hoyt, Rick Jackson, Catherine May, Anne Penney, and Virginia Russell for many friendly conversations and for needling me to the point of laughter, whenever I so desperately needed a laugh. "Catherine—Where's Harry?" I'm sorry. I should know better by now.

I would like to thank Dian Buchness for taking care of all of the administrative paperwork so that I could focus on the science. Dian always had a free moment (or 40 to 50 minutes) for me when I stopped by her office unannounced, and she always listened to my frustrations and triumphs. Thank you, Dian!

Angelo Di Bilio (aka Dottore) got me started on the rhenium-azurin project. From Dottore, I learned the practical aspects of the preparation of metal-modified proteins; he also trained me to be a competent EPR spectroscopist. I had a valued collaborator on the radical project, and we endured many of the anxieties and uncertainties that any project inevitably provides. Grazie, Dottore.

Brian Crane and Cristian Gradinaru were the collaborators on the crystal structure determination of the Re(His107) azurin. Brian was originally a postdoctoral colleague and had a hand in getting me started on the rhenium-azurin project, one of his many accomplishments at Caltech.

Michele McGuirl ($M^2$) had a tremendous influence on me during her time as a postdoctoral colleague. She patiently explained all of the biological steps that I had to go through to get protein and kept my eyes open to problems of biological interest and kindly gave me a tour of Yellowstone, a nice detour during a visit to her in Missoula.

Ivan Dmochowski and I spent three years as graduate student colleagues and in a few months I will be his postdoc at Penn. I eagerly look forward to this adventure.

Katrine Museth and I collaborated on the rhenium-azurin project. Katrine made all three of the His83 mutants starting with His107 plasmids; she and I got the cysteine mutant experiments going together.

Just over a year ago, Yen Hoang Le Nguyen joined the Gray Group and moved into the office with me. Yen and I had many interesting discussions, which provided necessary distractions while I was writing up my props and my thesis and she was writing up her candidacy. The last year of graduate school is the most exhilarating and stressful, but I could always count on Yen to put everything in the proper perspective. Thank you, Yen!!

Julia Lyubovitsky and I shared an office for four years. Many interesting conversations resulted, on various subject—life, science, psychology, language, and history (to name a few)—and I found Julia's frank perspective refreshing. Towards the end of our graduate experiences, we shared our office with a fourth person, Julia and Russ' soon-to-be one-year-old son David, who always and easily managed to get the attention of the third floor of Noyes and who, for some reason, always found it fascinating. Julia liked to remind me about how much smarter David is than I, a point that I could never argue against and that I am not likely to forget!

Kevin Hoke was a more senior graduate student when I joined the Gray Group and helped get a first-year started in the lab on the third floor of Noyes. He was always very generous with his time, and he patiently answered an endless list of my questions.

Jeremiah Miller was my resource for all the laser experiments whose results I present in this thesis. In addition, Jeremiah was an outstanding collaborator to have on the rhenium-azurin project and a great person to bounce (crazy) ideas off of, over the ever-present cup of coffee. I discussed every aspect of my project and my thesis with him and I appreciate his suggestions and insight, which improved both my thesis and my

graduate experience. The youthful Jeremiah made PhD before I did (by a day). It was an honor for me to work with him.

Pierre Kennepohl (aka Dr. Pierre) was worth the wait. During my entire career in chemistry, I have not met anyone with more similar interests or approaches in science to mine than Dr. Pierre. Even though we overlapped for just one year, I am forever in his debt for his unwavering and infectious enthusiasm for science and for his optimistic perspectives, both of which invariably cheered me up during the final stages of my stay in graduate school. Dr. Pierre and I worked on the thiyl radicals together (experimentally and theoretically); he also helped me with the theoretical calculations (and the understanding of their results) on the rhenium-coordinated imidazole radicals, which I present in this thesis. He reawakened my interest in ligand field theory and in the study of the electronic structures of inorganic complexes ("The buzz word is spin-orbit coupling. We don't know why, but it is."). He also very patiently listened to every complaint or frustration that I had, over the bottomless cup of tea or diet-coke or, when I was really down, 12 to 18 year old single-malt Scotch Whiskeys (for which I have become quite the connoisseur!). Special thanks go to Jen Love, for letting me get away with teasing Dr. Pierre endlessly. That was my way of continuously thanking him.

Lori Lee is an outstanding friend with whom I had many lengthy and memorable conversations while indulging in fine chocolates or while running to the Rose Bowl and throughout San Marino. From organic photochemistry to unraveling the meaning of various mysteries in the Big Picture, I learned from Lori's logical approach, which differs from mine, and benefited from all her points of view. Somehow, everything always

became clearer after running an idea by Lori, a mystery in itself to me, but a tribute to her for making me think from another perspective. Thank you, Lori!

Ramez El-Gammal and Sara Klamo are wonderful friends with whom I enjoyed many extensive conversations over the Middle Eastern feasts we prepared, and over the obligatory glasses (note the plural) of Arak and the plentiful supply of Turkish coffee. I thank them for their encouragement and levelheadedness (and runs through San Marino!), especially during the last few months when my life became the most stressful and hectic. Thank you, Ramez and Sara!

Cindy Quezada is Lady Luck. For the last year, Cindy and I motivated each other to finish our experiments and to write and defend our theses in time to walk in the 2003 commencement ceremony. We made it and we managed to have some fun along the way, with the humor always (and rightfully) at my expense. Earlier on, whenever I got colonies on plates of azurin mutants (and wanted to celebrate) or needed to run a gel, I wandered over to Cindy's corner in the Simon Labs (I pondered asking Mel for bench space!) and then proceeded to talk about what I was up to while she graciously listened. When I struggled, Cindy always came up with the right suggestions that I had overlooked, which always worked. I appreciate the perspectives, professional and otherwise, that Cindy gave me along with her encouragement during the final stretch of my graduate career. Lady Luck smiled upon me and for this I am grateful. Thank you, Cindy. Thank you.

I could never have come to this point in my life and career without the constant support, encouragement, and patience from my family in the U.S.A. and Lebanon: Dad, Mom, Jen, Tayta Faridi, Uncle George, Auntie Mary, Margaret, Linda, Georgia, Uncle Samir, Auntie Amal, Rick, Sam, Uncle Nicola, Tayta Zakia, Auntie MM, Uncle Kamal, Auntie Suzie, Uncle Maher, Auntie May, Nuha, Michel, Naji, Bassem, and Rhea. My parents especially, who have put up with me the longest, put up with extended and excessive stretches of time between my visits to them and with my venting phone calls at all hours of the day and night whenever I felt that I could no longer handle the pressures and stresses I put myself through. My sister always found clever ways to cheer me up and then was gracious and patient with me when I was overly exuberant. Visits with my Uncle George provided me with weekly escapes from Caltech and with the equanimity I needed to maintain the focus on my work and the proper perspective on my responsibilities in life. Except for me, no one in my family ever lost any sleep over the role of tyrosyl radicals in multi-step tunneling or over how a sulfinyl radical could have formed in a supposedly anaerobic EPR tube (to name two examples). Even when they wondered what I was studying, how I could it find it exciting, and why it consumed six years of my life, they always had confidence in me and in the path that I chose. The most difficult part of graduate school was the geographical separation I had to endure from the ones I love most. Because of this, I know how precious the moments are that I spend with them.

Finally, the year 2003 marks the Centennial of the birth of my paternal grandfather Amine. I never met him or my maternal grandfather Michel, yet the effects

of their lives and of their losses have been and are still visible on the generations who followed them in the last half-century. My greatest inspiration has come from my great-uncle William, who filled the role of grandfather. He was an outstanding teacher and scholar who set for his namesake nephew the highest possible standards with his integrity, diligence, patience, enthusiasm, and optimism.

***I DEDICATE THIS THESIS TO THE MEMORY OF UNCLE WILLIAM.***

## Table of Contents

## Abstract

Oxidative flash quench of $[Re(CO)_3(phen)(His)]^+$ generates a high-potential $[Re]^{2+}$ oxidant ($E°$ ($Re^{2+/+}$) = 2 eV v. NHE), which has been used to obtain rates of electron transfer of Cu(I) oxidation in rhenium-modified azurins. These rates are enhanced over the $[Ru(bpy)_2(im)(His)]^{2+}$ analogues ($E°$ $Ru^{3+/2+}$ ~ 1 eV), suggesting an alternate mechanism from driving force optimized, singe-step electron tunneling. To test whether other intermediates can be involved, oxidative freeze flash quench of the zinc(II) derivatives was undertaken. These experiments reveal that $[Re]^{2+}$ can produce the amino acid radicals of tyrosine and cysteine, as detected by EPR. The properties of these radicals in structurally well-defined protein microenvironments in *Pseudomonas aeruginosa* azurin mutants have focused, in particular, on the $g_1$ component of the g-tensor, which is sensitive to the strength of the hydrogen bond to the radical. The $g_1$ for Tyr48 radical, which resides in a completely hydrophobic pocket and is inaccessible to solvent, is found to be greater than the $g_1$ for the solvent exposed Tyr108 radical. This comparison could not be made for the cysteine radicals as Cys108 formed a sulfenyl radical upon oxidation; the Cys48 radical has been demonstrated to be a thiyl radical species and provides the EPR spectroscopic benchmark for a non-hydrogen bonded thiyl radical.

In azurin mutants without any tyrosine, tryptophan, or cysteine residues, oxidative flash quench results in another organic based radical. This radical is located on the histidine imidazole ring that is coordinated to the rhenium atom. DFT calculations suggest that the spin density resides mainly on the imidazole ring when it is deprotonated.

Corrected distances in the tunneling timetable to the imidazole ring from the copper atom predict an identical exponential decay in the electron transfer rates as for the ruthenium-labeled azurins. The rate enhancement is explained in terms of a "trivial hop" whereby $Re^{2+}$ rapidly oxidizes the non-innocent histidine ligand in a proton dependent process; the histidine radical in turn oxidizes the copper atom or tyrosine, cysteine, or tryptophan when zinc is present. This model explains *all* of the enhanced Cu(I) oxidation rates by $[Re]^{2+}$ and suggests that for Cu(I) oxidation in azurin, multistep electron tunneling through other amino acid radicals does not occur and that the observed radicals are generated in off-path processes.

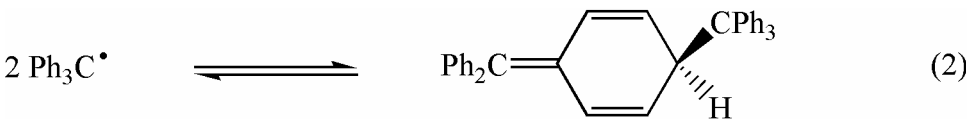
# Chapter 1

## Introduction

In 1900, Gomberg reported on his attempts to prepare hexaphenylethane.[1] He had proposed that he could reach the target molecule by adding silver metal to a solution of triphenylmethylchloride in benzene (Equation 1).

$$2\ Ph_3CCl + Ag \rightarrow Ph_3C{-}{-}CPh_3 + 2\ AgCl \qquad (1)$$

While the elemental analysis for carbon and hydrogen matched the predicted value, the physical and chemical properties of the product did not fit the expectation that hexaphenylethane, a saturated alkane, would be colorless and unreactive. Although Gomberg found that the crystals of the compound were colorless, the solutions were yellow-orange. The solution reacted with oxygen to give the triphenylmethyl peroxide dimer, with iodine to give triphenylmethyliodide, and hydrogen (on platinum metal) to give triphenylmethane, all of which were characterized. In addition, Gomberg found that the mystery compound was sensitive to acid and light and that it conducted an electric current in liquid $SO_2$. These observations led Gomberg to suggest that he had prepared an unsaturated species with a trivalent carbon atom, the triphenylmethyl *radical*, which became the first compound known to deviate from Kekulé's quadrivalent theory of carbon. Further preparations of various triarylmethyl radicals and other radicals along with the studies of their properties and reactivities were carried out by Gomberg and others, with Gomberg leading the field.[2-5] The structure of the colorless solid that Gomberg obtained was proposed in 1904 to be an unsymmetrical quinoid (Equation 2), but was not confirmed until 1968 by NMR (the history of these developments has been reviewed by McBride[6]).




(2)

A recent review of radical chemistry in the twentieth century provides a modern perspective on these early studies on radicals.[7]

In the 1930s, Michaelis investigated the reversible oxidation-reduction properties of quinols to quinones and determined that this two-electron reaction can be carried out in two sequential one-electron steps through a stable semiquinone intermediate (Figure 1).[8,9] Furthermore, Michaelis speculated on the nature and role of radicals (especially semiquinones) in the oxidative catalytic processes of enzymes and also on the role of the protein medium for stabilizing or destabilizing the resonance structures of the radicals.[10-12] The techniques available to Michaelis for studying radicals were potentiometric (by redox titrations), magnetic (susceptibility studies), and colorimetric, all three of which could only be applied to stable radicals. With the development of electron paramagnetic resonance (EPR) spectroscopy in the 1940s and 1950s and its early applications to biological samples,[13] a new technique emerged for studying radicals. In addition, frozen samples could also be irradiated with ionizing γ-rays and the stability and reactivity of the generated radicals could be studied as a function of temperature (4-200 K). The first experiments were performed on various biological tissues,[14,15] which gave resonances centered around the free electron g value ($g_e$ = 2.0023). Gordy extended these studies to irradiated solutions, powders, and single crystals of the amino acids, peptides, and proteins, thus beginning the study of the properties of amino acid radicals.[16-20]

In 1973, Ehrenberg and Reichard[21] reported the EPR spectrum (Figure 2) of a stable organic-based radical in the resting state of the B2 protein ribonucleotide reductase (RNR) from *E. coli*. The RNR system from *E. coli* consists of two separate proteins that are responsible for the reduction of ribonucleotides to deoxyribonucleotides. The B2

(also called R2 in the current literature) protein is a di-iron enzyme, which activates molecular oxygen to produce a stable organic radical. The reduction of ribonucleotides takes place in the B1 (also called R1) subunit and its initiation (by a thiyl radical, *vide infra*) is dependent on the presence of the B2 subunit and on this radical[22] (the crystal structures of both proteins have been solved separately, R2 in 1990,[23] and R1 in 1994,[24] and models have been proposed,[25] but not confirmed, for complex formation). Subsequent work confirmed that the stable radical in the B2 protein is tyrosyl.[26,27] This discovery, one of the many surprises in biochemistry,[28] prompted the search for other amino acid radicals in proteins, and led to extensive studies to elucidate the roles of these intermediates in enzyme catalysis.[29-36]

In addition to the tyrosyl radical in ribonucleotide reductase, two tyrosyl radicals have been found in photosystem II. A EPR signal assigned as D·+ was first reported in 1956 in irradiated chloroplasts.[37] In the 1970s, a similar EPR signal, called Z·+, was detected.[38,39] This radical species was found to be required for catalytic activity in the oxidation of water and is much less stable than the catalytically inactive D·+. In 1987, Babcock presented a study in which the two radicals of PSII were assigned to tyrosine.[40] Subsequent work on the kinetic and spectroscopic properties of these tyrosine residues (now called TyrZ and TyrD for Z and D, respectively) led to a metalloradical mechanism in PSII for the oxidation of water to molecular oxygen whereby the TyrZ reduces the oxidized chlorophyll special pair (P680+) in a proton dependent process. In turn, the radical on TyrZ proceeds to oxidize the manganese cluster in the process of water oxidation. Babcock's monumental work[41-47] on the tyrosyl radicals in PSII also looked into the proton dependence of the electron transfer oxidation and reduction processes at

TyrZ during photosynthetic water oxidation as an example of proton coupled electron transfer. In addition to PSII, prostaglandin H synthase has also been found to require a tyrosyl radical intermediate in the oxygenation of arachidonic acid to prostaglandin G2.[48-50]

Tyrosyl radicals were the first amino acid radicals to be discovered and have been the most extensively studied.[51,52] In addition, some enzymes such as the copper amine oxidases can modify a particular tyrosine residue to topa quinone (Michaelis' quinone is now protein based rather than exogenous), which is catalytically active.[53-55] Galactose oxidase has also been found to contain a tyrosyl radical intermediate in catalysis.[56-58] This residue, like the topa quinone, is post-translationally modified via a cross link of cysteine to tyrosine.[59] The phenol end of the tyrosine residue is also coordinated to copper. Thus, a two-electron redox reaction can occur, with one equivalent coming from copper and the other from the non-innocent tyrosine ligand. Examples of other novel cofactors and post-translational modified amino acid residue in proteins have been reviewed recently.[60,61]

Subsequent to the discovery of tyrosyl radical intermediates in enzyme catalytic processes, tryptophan radicals were identified in cytochrome c peroxidase (spin-coupled to a ferric heme),[62-65] DNA photolyase (free),[66-70] as well as various engineered tryptophan mutants of RNR.[71-75] Except for a commentary[76] following the initial discovery of tryptophan radicals, this field has not been reviewed.

In 1989, Boussac and coworkers observed an organic radical intermediate EPR signal in the S2 → S3 step in the photocycle of $Ca^{2+}$ depleted photosystem II.[77] As the S3 signal is normally undetectable by EPR, this curious result raised questions about the

nature of the radical. Oxidized amino acids that interact magnetically with the manganese complex were considered as possible candidates for the radical. On the basis of an optical spectrum of this modified S3 state, a histidinyl radical was proposed to correspond to the signal occurring in the modified S3 state.[78] Further investigation[79] challenged this assignment with the suggestion that the radical is TyrZ, which prompted a reply[80] containing analyses reinforcing the original tentative assignment as more probably a histidinyl radical than tyrosyl radical. Since these experiments, the histidinyl radical has not since been revisited as an intermediate in PSII. However, a recent report suggests histidinyl radical formation in a copper-zinc superoxide dismutase (Cu/Zn SOD) as detected by 2-methyl-2-nitrosopropane spin trapping.[81] This study followed the characterization of 2-oxo-histidine that was generated selectively at one histidine (His118) in bovine Cu/Zn SOD upon addition of $H_2O_2$ to the enzyme.[82] The spin-trapped adduct on His118 (Figure 3) provided another validating data point for Michaelis' original hypothesis that two-electron oxidation-reductions proceed in one-electron steps, even though the overall mechanism of formation of 2-oxo-histidine has not yet been established.

In the 1980s, while working on pyruvate formate lyase (PFL), an EPR doublet with a 15 G separation was observed as a catalytic intermediate.[83,84] This signal collapsed into a singlet in deuterated buffer; the radical was assigned to glycine, providing the first example of a Cα backbone radical,[85] whose α-proton is exchangeable with deuteron, explaining the conversion of the EPR doublet to a singlet. In addition, PFL loses all activity in the presence of oxygen and subsequent cleavage occurs on the polypeptide chain at the N—Cα bond of the glycine.[85] While studying the mechanism of

oxygen inactivation of PFL, Kozarich and coworkers presented evidence for the glycyl peroxyl radical, which is expected to be responsible for backbone cleavage, and also a long-lived cysteine-based sulfinyl (RSO·) radical.[86,87] Glycyl radicals have since been assigned in the Class III ribonucleotide reductase[88] and in benzylsuccinate synthase,[89] which derive from anaerobic organisms. In all three known cases, the glycyl radicals are generated by hydrogen atom abstraction by 5'-deoxyadenosyl free radical following homolytic cleavage of S-adenosylmethionine.[90] The glycyl radical, in turn, abstracts a hydrogen atom from an adjacent cysteine residue, which is the catalytically active species. A more detailed review of glycyl radical enzymes has recently appeared.[91]

In the course of the study of the three classes of ribonucleotide reductase, Stubbe found that all three enzymes required a cysteine residue for proper function.[92] The proposed mechanism has a cysteine thiyl radical abstracting H-atoms from ribonucleotides in the initiating step and then providing H-atom reducing equivalents in the final step, making the cysteinyl radical catalytic (the formal reducing equivalents are provided by two other cysteines going to cystine). Here, Stubbe found an example of a divergent evolutionary process (Figure 4) in which reactivity was centered around cysteinyl radicals that were generated in different ways. In class I, a tyrosyl radical is presumed to initiate a radical chain of oxidations to get to the catalytic cysteine; in class II, adenosine cobalamin produces the thiyl radical; in class III, a glycyl radical oxidizes cysteine. Stubbe has observed the thiyl radical in the class II RNR and found that even though it is spin-coupled to the cob(II)alamin, deuteration of the β-protons of cysteine lead to changes in lineshape, due to the hyperfine coupling to I = 1 nuclei compared to I = ½ nuclei and their different nuclear magnetic moments.[93] To date, this is the only thiyl

radical that has been detected, although it is not the only thiyl radical catalytic intermediate in an enzyme process. Lassmann recently reported the detection of thiyl and sulfinyl radicals in γ-irradiated RNR[94,95] and Graslund[96] has demonstrated sulfinyl radical by dioxygen activation in RNR by introducing a cysteine residue near the di-iron center of the R2 protein.

With the demonstration of catalytically active amino acid radicals in enzyme catalysis, it became necessary to assess the redox properties of these radicals and their role in long-range electron transfer. Dutton and coworkers have inserted tyrosine and tryptophan residues into protein maquettes and have studied their redox properties by differential scanning calorimetry.[97] With theoretical methods to treat the effective dielectric around these radicals, they have established the dependence of the electrochemical properties of these radicals on their protein microenvironment. High-frequency EPR has been used by Un,[98,99] Griffin,[100] and Lendzian[74] to determine accurate values of the g-tensor components in tyrosyl and tryptophan radicals (a recent review[101] of high-frequency EPR and its applications to bioinorganic chemistry provides more extensive references to the wide range of applications of this technique to organic radicals and to metal ions and clusters). The $g_1$ component is sensitive to the strength of the hydrogen bond to the tyrosyl radical and can provide a handle on the polarity of the protein microenvironment; and the $g_2$ and $g_3$ components have been found to remain essentially constant for all tyrosyl radicals measured to date.

In their work on electron transfer in proteins, Gray and coworkers have established the distance and driving-force dependences on oxidations by $Ru^{3+}$ in ruthenium-modified proteins.[102-104] From these studies, estimates of the reorganization

energy have been made. When the driving force equals the reorganization energy, the electron transfer rate is activationless (and thus fastest). Further increases in the driving force can lead to inverted rates, first proposed by Marcus,[105,106] where the rate of the electron transfer step decreases with increasing driving force. Gray and coworkers reacted *fac*-$[Re^{I}(CO)_3(phen)(OH_2)]^+$ with azurin (substituting the aquo for a single surface histidine) with the intention of studying electron transfer at higher driving forces than those afforded by ruthenium complexes: $E° Re^{2+/+} = 2$ eV; $E° Ru^{3+/2+} \sim 1$ eV.[107] The possible outcomes include: slower rates, due to the inverted effect; faster rates due to multistep tunneling through amino acid radical intermediates; enhanced rates due to the near resonance of the 2 eV rhenium acceptor with the 2.2 eV bridge energy (measured for the oxidation of toluene and applied to phenylalanine).[108] The last of these models is the tunneling energy effect first proposed by McConnell[109] and developed further by Beratan.[110] Oxidation rates were found to be enhanced and therefore a systematic study was undertaken to determine whether or not amino acid radicals can be produced by $Re^{2+}$ and whether they have a role in multistep electron tunneling.[111] A preliminary communication has appeared describing the formation of tryptophan and tyrosyl radicals in rhenium modified copper proteins.[112]

This thesis explores the nature of photogenerated $[Re]^{2+}$ and the properties of tyrosyl and cysteinyl radicals in rhenium-modified azurins and their effects on the rate of oxidation of Cu(I). Tryptophan radicals in rhenium-modified azurins and $[Re]^{2+}$ model complexes are treated in depth in the thesis of Jeremiah Miller.[113]

**Figure 1**: The two-electron oxidation-reduction conversion of quinone to hydroquinone proceeding through a stable semiquinone intermediate.

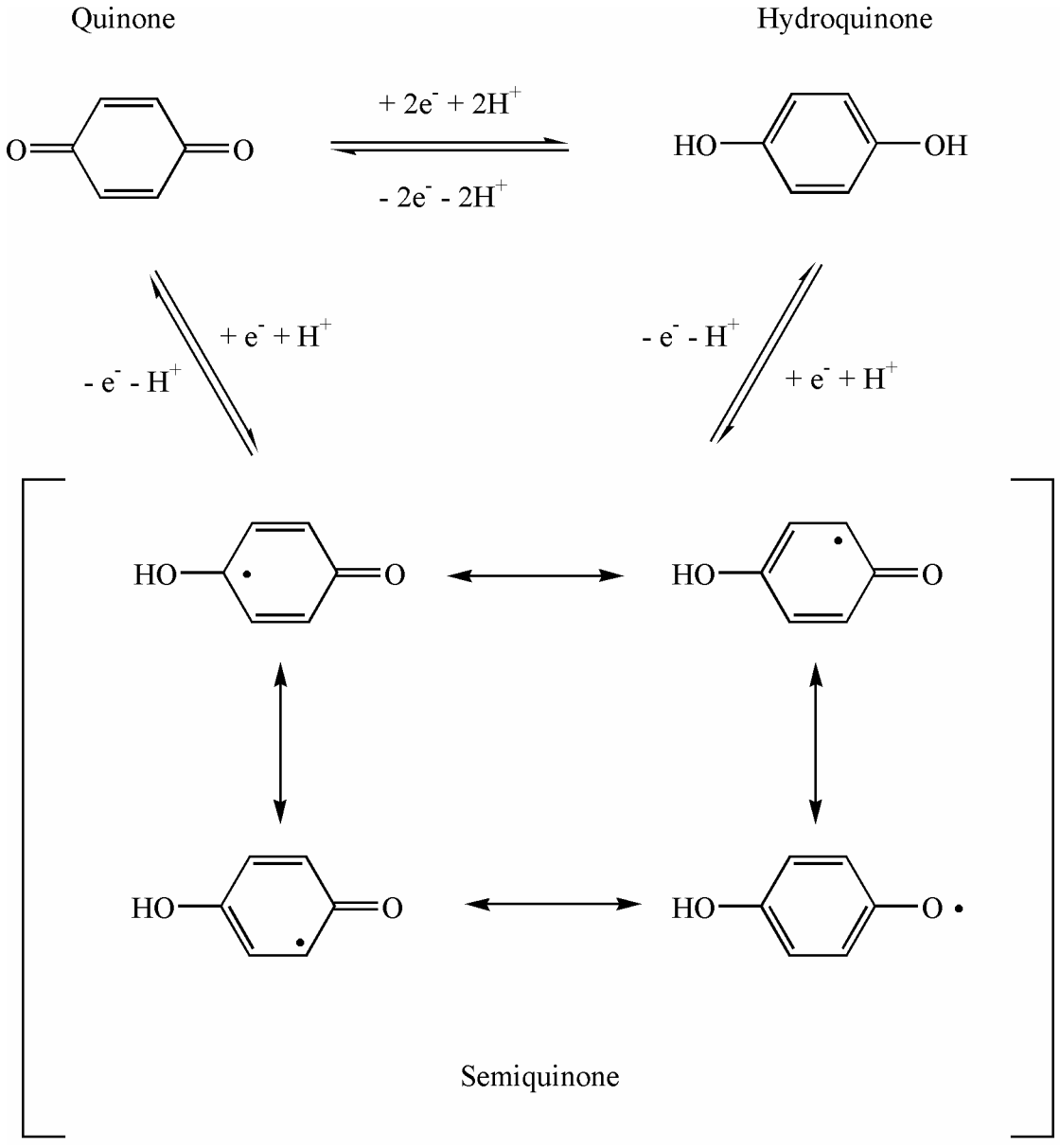

Quinone
Hydroquinone
+ 2e- + 2H+
- 2e- - 2H+
+ e- + H+
- e- - H+
- e- - H+
+ e- + H+
HO
O
OH
Semiquinone

**Figure 2**: EPR spectrum of the stable organic radical species in RNR (adapted from Ehrenberg and Reichard).[21]

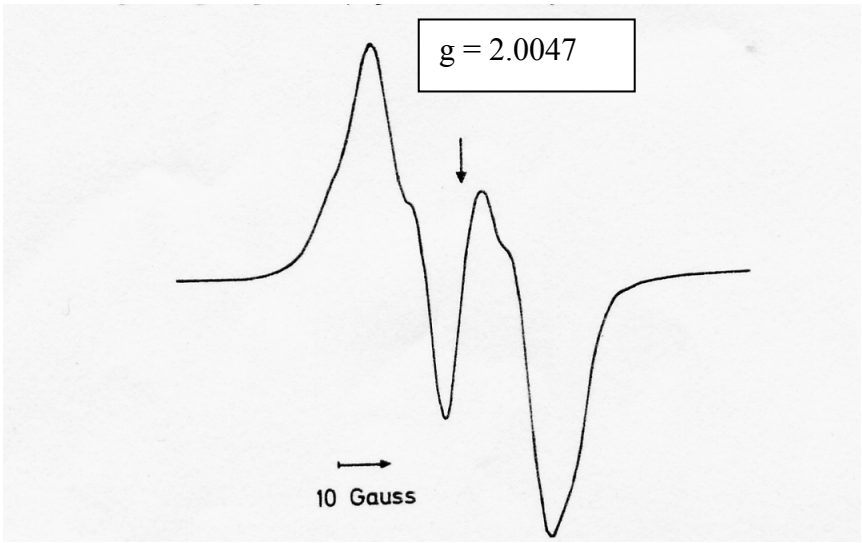

g = 2.0047
10 Gauss

**Figure 3**: Possible scheme of 2-oxo-histidine formation through a histidinyl radical.

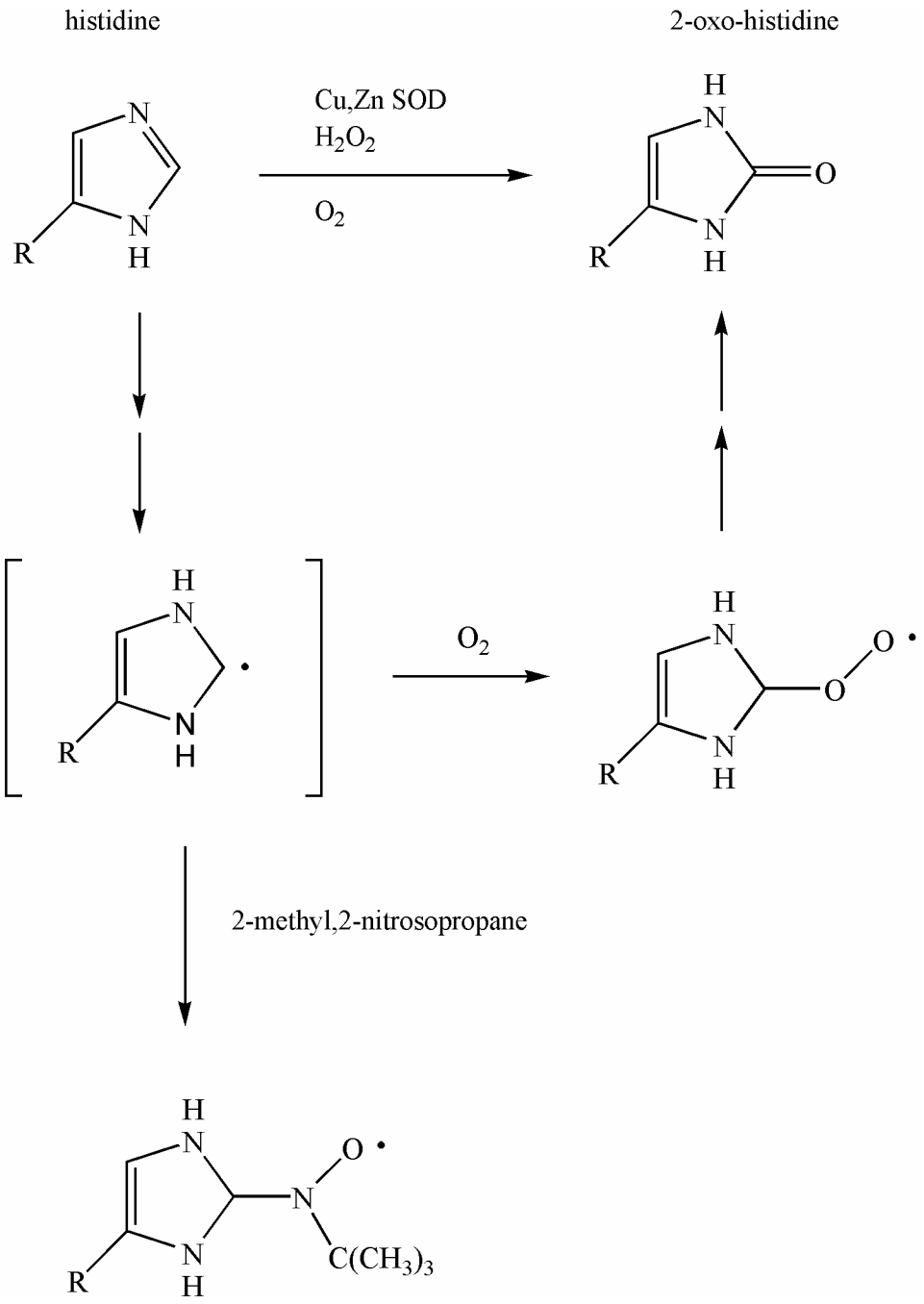
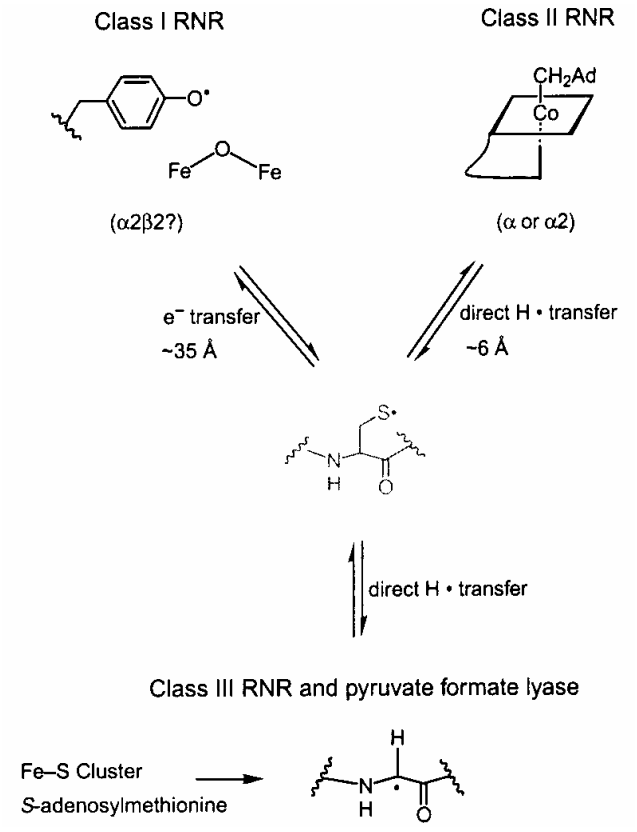

histidine
2-oxo-histidine
Cu,Zn SOD
$H_2O_2$
$O_2$
$O_2$
2-methyl,2-nitrosopropane
$C(CH_3)_3$

**Figure 4**: Divergent evolution illustrated by the three classes of RNR.[92]


Class I RNR
Class II RNR
O•
Fe
O
Fe
CH2Ad
Co
(α2β2?)
(α or α2)
e⁻ transfer
~35 Å
direct H • transfer
~6 Å
S•
N
H
O
direct H • transfer
Class III RNR and pyruvate formate lyase
Fe–S Cluster
S-adenosylmethionine
H
N
H
O

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# Chapter 2

## Histidine Radicals Coordinated to Rhenium

## Introduction

Electron transfer reactions in metalloproteins have been studied extensively by introducing ruthenium complexes to surface amino acid residues—in particular, single surface histidines.[1-3] In their ground states, the ruthenium complexes are kinetically and thermodynamically unreactive to redox with the donor or acceptor of the metalloprotein. However, following light excitation into their metal-to-ligand charge transfer (MLCT) excited states, these ruthenium complexes become powerful oxidants and reductants (Figure 1). In these photoinduced experiments, the rates of electron transfer that can be measured are limited to the rates that are faster than those of the excited state emission ($k_{em} = 1.7 \times 10^6\ s^{-1}$ for $Ru(bpy)_3^{2+*}$).[4,5]

To access a greater temporal window, the flash quench method is used.[6] In this approach, the excited states react with exogenous oxidative[7,8] or reductive quenchers[9-11] to yield ground state oxidants or reductants respectively (Figure 2). The fastest electron transfer rate that can be measured is limited by the rate of excited state quenching ($k_q$), while the slowest depends on the stability of ground state oxidant or reductant in water. Thus the range is defined by $k_q > k_{ET} > k_{gr}$, which spans from nanoseconds to seconds. From these studies, complemented with site-directed mutagenesis to insert histidine residues at different surface sites, a reliable picture can be obtained of the distance dependence on the electron transfer and thus the role of the intervening medium, which in turn have led to the proposed electron tunneling pathways model.[12-14] In addition, with the flash quench system, variation of the substituents of the ruthenium complex changes the driving force of the electron transfer process. Thus, the dependence of the driving

force on the electron transfer has been monitored and experimental values for the reorganization energy have also been obtained.[3]

The oxidative flash quench method for studying electron transfer in proteins has been more thoroughly explored and applied in proteins (cytochrome c,[6] azurin,[15] plastocyanin,[16] HiPip[17] and DNA[18]) than the reductive flash quench method (which has been applied in cytochrome c[19] and cytochrome P450[20] and protein triggered folding[21-23]). In the oxidative flash quench experiment, $Ru^{3+}$ is produced. For $Ru(bpy)_3^{3+}$, the redox potential is known ($E^o$ $Ru^{3+/2+}$ = 1.26 eV versus NHE in water)[24-26] and its optical[27,28] and EPR spectra[29,30] have been obtained ($Ru^{3+}$ is stable in water on the order of minutes, but can be reduced to $Ru^{2+}$, presumably by the oxidation of water). The absorption and luminescence properties[27,31,32] of $[Ru(bpy)_3]^{2+}$ provide the complete spectroscopic handle for the ruthenium system for time-dependent optical spectroscopic measurements.

The blue copper protein azurin, from *Pseudomonas aeruginosa*, is one of the model protein systems in which oxidation have been studied by flash-quench of surface attachment of ruthenium complexes. Azurin is a small protein (128 residues) that has a beta sheet structure of eight antiparallel strands in which a copper atom is embedded.[33-35] The single copper atom is coordinated by two histidines (46, 117) and one cysteine (112) in a trigonal plane; a methionine (121) sulfur and a backbone carbonyl oxygen of glycine (45) are weakly coordinated axial ligands.[36] This beta sheet structure has made azurin an outstanding model for the study of electronic coupling of the beta sheet.[15] These results complemented the studies of electronic coupling in alpha helical structures.[3] Along with the studies of electronic coupling in water[37] a master tunneling timetable was constructed

(Figure 3) clearly showing the exponential decay of the rate of electron transfer depending on distance.

In 1995, Connick *et al.* reported the preparation and characterization *fac*-$[Re(CO)_3(phen)(im)]^+$ and its preliminary electron transfer properties when surface labeled on azurin (where im is now a surface histidine residue).[38] The photophysical properties of related $Re(CO)_3(diimine)(X)$ molecules (where X=Cl, and Br) were first investigated by Wrighton in the mid-1970s and were found to possess luminescence properties quite similar to the ruthenium polypyridyl systems.[39-41] Since the original studies, A. Vleck Jr. and coworkers have carried out an extensive study of the excited states of these complexes with various diimines and an extensive list of X ligands (such as H, $CH_3$, pyridine, etc).[42-45] Bard pursued one study on the electrogenerated chemiluminescence properties of $Re(CO)_3(phen)Cl$.[46]

The complex $Re(CO)_3(phen)(im)$ exhibits a quasi-reversible oxidative wave in acetonitrile (and nitromethane[47]) that gives an $E^o$ for $Re^{2+/+}$ of 1.85 eV versus the Ag/AgCl reference electrode. This rhenium systems offers the possibilities for exploring inverted region behavior, tunneling energy effects, and multistep tunneling. In 1999, the oxidative flash quench rate enhancement for ReAz as compared to RuAz was reported for the His107 rhenium modified azurin.[48] Multistep tunneling through a tyrosyl radical (residue 108) was implicated (Figure 1, Chapter 3, this thesis) although no mention was ever made of the detection of $[Re(CO)_3(phen)(His)]^{2+}$. We therefore ask: what is the nature of $[Re]^{2+}$ in the flash quench oxidized system and what is its role in electron transfer oxidation of Cu(I) azurin?

Before addressing this question, we would like to note the rarity of mononuclear complexes of $Re^{2+}$ (by contrast, the $Re^{2+}Re^{2+}$ multiply bonded dimers are well known and have been extensively studied.[49] Ballhausen[50] noted in 1962 that: "With the electronic structure $[Xe](5d)^5(6s)^2$, rhenium should resemble Mn, and this expectation is found to be justified. The greatest difference between the two elements is that Re++ is nearly unknown." At that time, no $Re^{2+}$ complex had been prepared and it was not until 1973 when Chatt and coworkers reported the first mononuclear complex of $Re^{2+}$ as produced by chemical oxidation of a $Re^{+}$ dinitrogen complex.[51] Very few $Re^{2+}$ complexes have since followed, although Harman and coworkers have recently demonstrated the preparation of $[Re(bpy)_3]^{2+}$, TpRe(phen)Cl, and related complexes.[52,53] It is interesting to note the chemical similarities of $Tc^{2+}$ and $Re^{2+}$, which are without parallels to $Mn^{2+}$ chemistry. One particular example is the absence of the *fac*-$\{Mn(CO)_3\}$ moiety, as opposed to the extensive studies of the rhenium and technicium analogs.

In this chapter, we attempt to understand the properties of $[Re(CO)_3(phen)(His)]^{2+}$ in *Pseudomonas aeruginosa* azurin mutants as generated by the flash quench oxidation of the $Re^{+}$ excited state. The study of $Re^{2+}$ model complexes is addressed by Jeremiah Miller in his thesis.[47]

**Materials and Methods**

The two mutant azurins that are described in this chapter are (1) W48F/Y72F/H83Q/Q107H/Y108F and (2) W48F/Y72F/Y108F. The preparation of these mutants, the flash quench the rhenium-modified proteins, the EPR detection of flash

quench products, and the DFT methods of computation for $[Re]^{2+}$ are described in the Appendices. The results of the DFT calculations are applicable to the study of the $[Re]^{2+}$ species in the rhenium-labeled azurins and in the $[Re]^{2+}$ model systems generated by oxidative flash quench.[47] In this thesis, the results will be discussed in the context of the $[Re]^{2+}$ species in the protein. The complementary analysis of the model chemistry is treated in the thesis of Jeremiah Miller.[47]

**Electron Transfer.** Electron transfer kinetics of $Cu^{+}$ oxidation for both mutations were measured on the Nanosecond 1 (NS-1) setup in the Beckman Institute Laser Resource Center. Samples were prepared in 25 mM potassium phosphate, buffered to pH 7, and contained 50 μM rhenium-azurin and 5 mM $Ru(NH_3)_6^{3+}$. The azurins had been reduced previously to $Cu^{+}$ by 1 mM solution of sodium dithionite. Dithionite was removed by gel-filtration (PD-10, Pharmacia).

**Results**

**Electron Transfer**. We pursued the flash quench oxidation of Cu(I) azurin in the rhenium modified His107 mutant to compare with the published ruthenium rates. The transient absorption spectrum monitored at 632.8 nm shows an enhanced rate for the oxidation of Cu(I) by "$Re^{2+}$" (Figure 5). Rates of $Ru^{3+}$ oxidation of Cu(I) and the analogous "$Re^{2+}$" oxidations are plotted in Figure 6 as a function the rhenium to copper distance.

**EPR**. Oxidative flash quench of the Re His107 zinc azurins gave the X-band spectra shown in Figures 7. The effective g-values were found to be 2.003. A high-

frequency, high-field EPR spectrum was obtained on the product of the His107 flash quench oxidation (Figure 8) and confirms the effective g-value found by X-band. We were unable to simulate these spectra into their g-tensor and hyperfine (A) tensor components.

We would like to mention an ongoing collaboration with Professor A. Vlcek of the University College in London on the nature of the excited states in the Re(His107) azurin. The excited state dynamics of Re(His107) in the Cu(I) and Cu(II) forms of azurin were studied along with the Re(Im) model complex (Dr. Angelo Di Bilio and Professor Vlcek). These studies demonstrate that the charge transfer is rhenium to phenanthroline in character; no imidazole oxidation in the charge transfer is observed (A. Vleck, personal communication).

**X-Ray Crystallography.** The X-ray structure of the rhenium-labeled W48F/Y72F/H83Q/Q107H/Y108F zinc azurin was solved by Mr. Cristian Gradinaru in the laboratory of Professor Brain R. Crane at Cornell University using protein that was sent from Caltech. The structure was found to be similar to the wild-type and other ruthenium, rhenium, and osmium labeled azurins.[35]

**Discussion**

In the two mutant proteins studied in this chapter, all tyrosines and tryptophans were removed from the protein, eliminating them as possible oxidation products. Phenylalanines are abundant although our radicals bear no resemblance to the benzyl radicals (in addition, the potential to oxidize toluene was reported to be 2.2 eV v. NHE,[54] which is above the oxidizing power of "$Re^{2+}$" as determined by cyclic voltammetry in

acetonitrile and nitromethane). A glycyl radical, which is based on the carbon backbone, would be expected to give a narrow EPR signal, split into a ~15 G doublet by an exchangeable alpha proton. The EPR spectrum of the Re(His107) oxidation product in deuterated phosphate buffer was found to be identical to its protic counterpart. No post-translational modifications of azurin prior to the photochemical experiment have ever been observed as demonstrated by mass spectroscopy (this is shown for the cysteine mutant proteins in Chapter 4 of this thesis).

We interpret our results to indicate the formation of rhenium coordinated imidazole radials formed after flash quench oxidation of the MLCT excited state. The width of the EPR signal (160 gauss in the X-band EPR) is comparable to those found in irradiated imidazoles. We were unable to obtain optical spectra of these radicals because their most intense peak (~30000 $cm^{-1}$, with epsilon ~5000 $M^{-1}cm^{-1}$)[55] would be found under the intense rhenium to phenanthroline charge transfer band. Hyperfine structure is not observed due line broadening induced by hyperfine coupling to rhenium, whose two isotopes, $^{185}Re$ and $^{187}Re$, which are 63% and 37% naturally abundant, respectively, have nuclear spins of I=5/2. DFT calculations substantiate this proposal as the wavefunction for $[Re]^{2+}$ is found to have significant spin density on both the imidazole and the rhenium. Remarkably, this shift in spin density is dependent on the protonation state of the imidazole. In the deprotonated form, the spin density resides mainly on the imidazole (80%), while in the protonated form, the spin density resides mainly on the rhenium (80%). We are currently exploring the nature of the electronic structure in these rhenium species based on the rotation of the imidazole. F.A. Walker and coworkers have pursued such a model for the co- and counter-rotation of bis-imidazole ligands in hemes using a

theoretical approach that they have tested experimentally with ESEEM.[56,57] We suggest that the redox active species in Cu(I) oxidation is the rhenium-coordinated imidazole radical, whose proton dependent redox potential would be less than the 2.0 eV of $Re^{2+}$ and more in line with the potentials obtained from model imidazoles by pulse radiolysis (~1.3 eV).[58] This model can explain the rate enhancement as a "trivial hop" by oxidation of the ligand, thus shortening the distance to the Cu atom. By correcting the rhenium to copper distances in the tunneling timetable to the rhenium to histidine imidazole ?-carbon, all of the rhenium rates fall on the ß=1.1 $Å^{-1}$ line (Figure 9).

In pulse radiolysis studies, the pKa of the imidazole radical was found to be in the range of 5-7.[58] Our experiments were conducted at pH 7, which makes it likely that *both* the $Re^{2+}$ and the $Re^{+}$(His•) species could be present and that they both could have participated in the oxidation of Cu(I). The faster phase is assigned to the His radical oxidation of Cu(I). We have not yet been able to assign the slower phase in the biphasic exponential, although we suspect that the slower phase, which still has an enhanced rate, may correspond to McConnell's tunneling energy effect, as the energy of the acceptor, $Re^{2+}$ (2 eV) is nearly in resonance with the bridge (2.2 eV, the oxidation potential of toluene, a model for phenylalanine). Further studies of the oxidation of Cu(I) must be conducted in order to test whether the proposed model is viable.

The idea of a radical ligand coordinated to a metal is not new. Gray and coworker first proposed ligand centered oxidations in Ni dithiolene complexes in the 1960s.[59] These ligands and others were termed non-innocent due to their involvement in redox and are excellent examples of complexes that highly covalent. The interest in non-innocent ligand chemistry did not advance until the 1990s, when Wieghardt and coworkers made a

series of complexes with the purpose of obtaining ligand centered oxidations. These studies followed up a proposal that in galactose oxidase, the two reducing equivalents in the oxidation of galactose came from Cu(I) and the phenol of tyrosine which is coordinated to the same Cu. Wieghardt's group has made complexes with coordinated phenoxylato, anilato, and phenylthiolato ligands whose oxidations can be assigned unambiguously to those ligands.[60] Wolfgang Kaim[61] (copper semiquinone) and Dan Stack[62] (galactose oxidase models) and their respective coworkers have also advanced various model systems containing non-innocent ligands. Representative studies of non-innocent ligands in proteins include galactose oxidase[63-65] and the amine oxidases. [66,67]

The most extensive EPR studies have been on organic radicals[68] and on transition metal complexes.[69,70] In the former case, the Huckel approach has served as an appropriate starting point for understanding their electronic structures,[71] while in the latter, it is ligand field theory.[72] However, there is no adequate theory to describe the electronic structures of metal coordinated radicals, which has proved to be a limitation on the study of non-innocent ligands

**Figure 1**: Scheme for the photoinduced oxidative and reductive electron transfer reactions in proteins.

$k_f$: rate constant for forward electron transfer

$k_b$: rate constant for back electron transfer (recombination)

$k_{em}$: excited state decay ($\tau = 1/k_{em}$ = the excited state lifetime = 600 ns for $[Ru(bpy)_3]^{2+}$*.

$M^{Ox}$: oxidized redox cofactor in protein

$M^{Red}$: reduced redox cofactor in protein

In this scheme, $[Ru]^{n+}$ denotes $[Ru(bpy)_3]^{n+}$ or more appropriately in the protein $[Ru(bpy)_2(im)(HisX)]^{n+}$

bpy: 2,2'-bipyridine

im: imidazole

His: histidine

X: amino acid residue

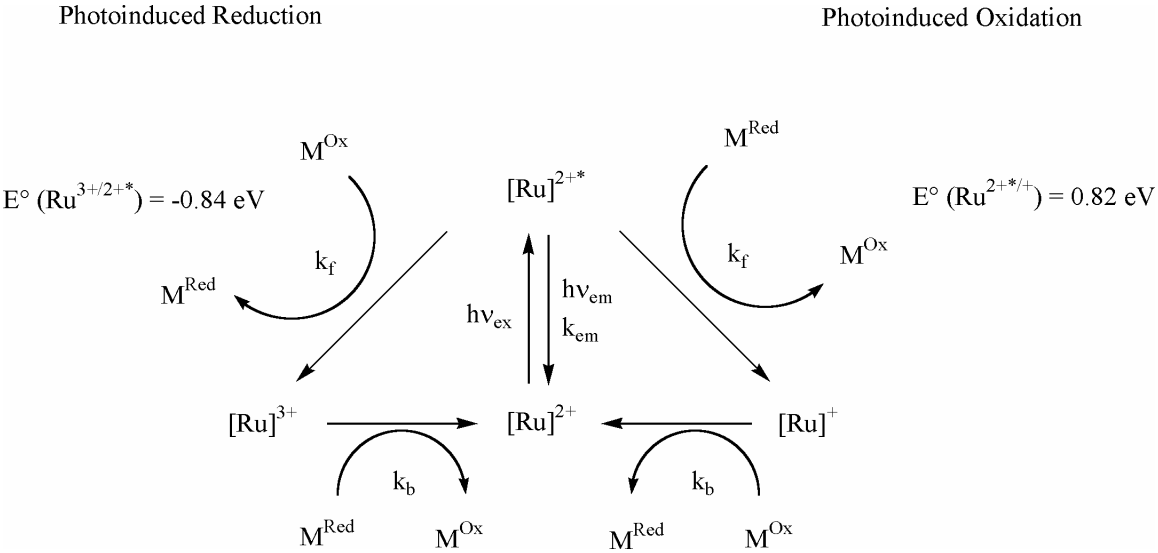

Photoinduced Reduction
Photoinduced Oxidation
E° (Ru3+/2+*) = -0.84 eV
E° (Ru2+*/+) = 0.82 eV
MOx
MRed
kf
[Ru]2+*
hνex
hνem
kem
MRed
kf
MOx
[Ru]3+
[Ru]2+
[Ru]+
kb
kb
MRed
MOx
MRed
MOx

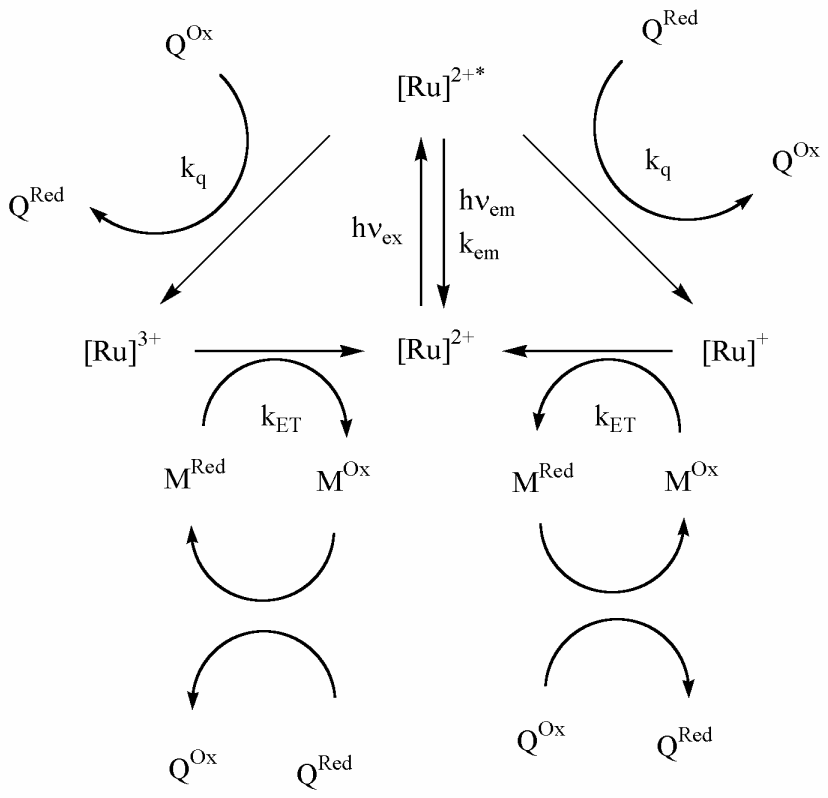
**Figure 2**: Oxidative and reductive flash quench scheme for electron transfer in proteins. Abbreviations are the same as in Figure 1.

Frequently used oxidative quenchers are: methyl viologen, $[Ru(NH_3)_6]^{3+}$ (reversible), $[Co(NH_3)_5Cl]^{2+}$ (irreversible).

Frequently used reductive quenchers are: $[Mo(CN)_8]^{4-}$, $[W(CN)_8]^{4-}$, $Eu^{2+}$, p-methoxy-N,N-dimethylaniline.

Oxidative Flash Quench

Reductive Flash Quench




$E° (Ru^{3+/2+}) = 1.26$ eV

$E° (Ru^{2+/+}) = -1.28$ eV

**Figure 3**: Master tunneling timetable for biological electron transfer reactions (adapted from Ponce *et al.*).[37]

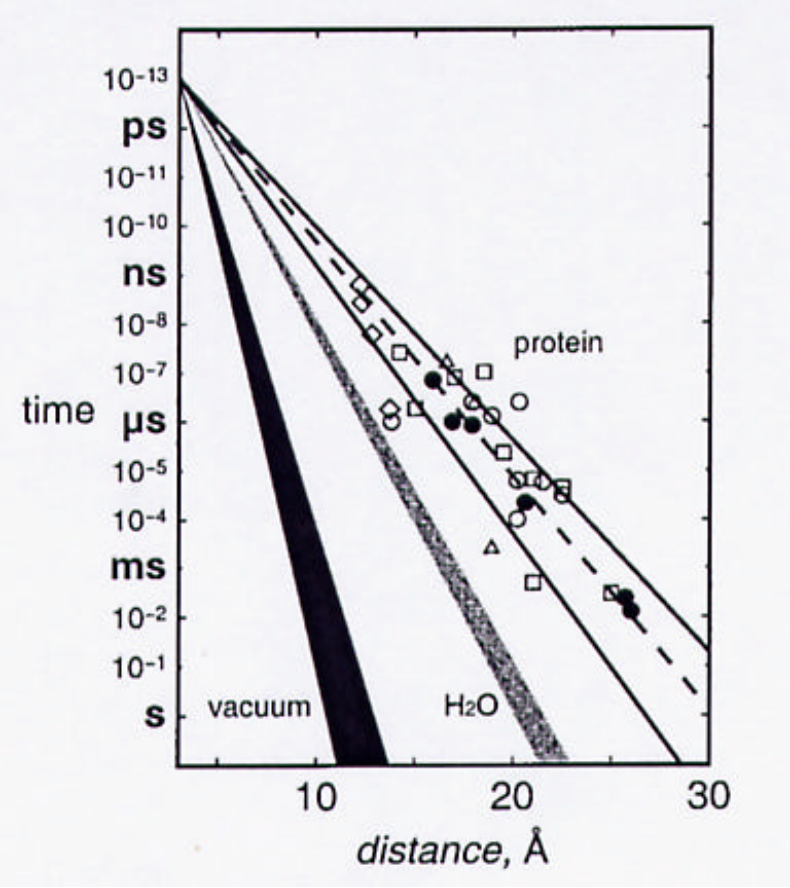

time
10⁻¹³
ps
10⁻¹¹
10⁻¹⁰
ns
10⁻⁸
10⁻⁷
µs
10⁻⁵
10⁻⁴
ms
10⁻²
10⁻¹
s
protein
vacuum
H₂O
10
20
30
distance, Å

**Figure 4**: Modified Latimer diagram for $Re^{+}$.

$[Re]^{n+}$ is taken to be $[Re(CO)_3(phen)(im)]^{n+}$.

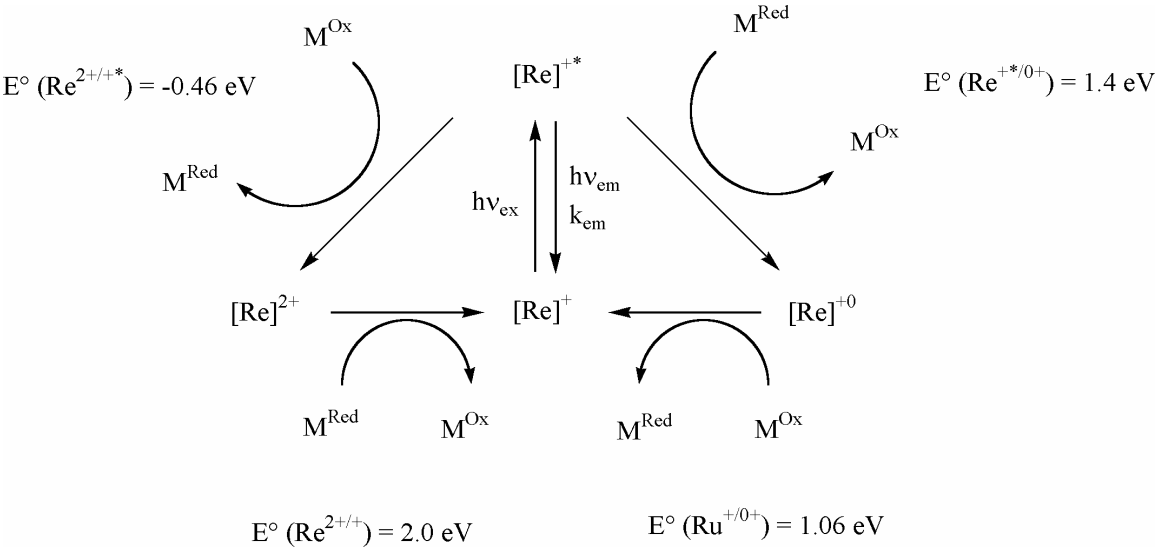

E° (Re2+/+*) = -0.46 eV
E° (Re+*/0+) = 1.4 eV
MOx
MRed
[Re]+*
MRed
MOx
hνex
hνem
kem
[Re]2+
[Re]+
[Re]+0
MRed
MOx
MRed
MOx
E° (Re2+/+) = 2.0 eV
E° (Ru+/0+) = 1.06 eV

Figure 5: Transient absorption spectrum of the flash quench oxidation of Cu(I) in the Re(His107) azurin.

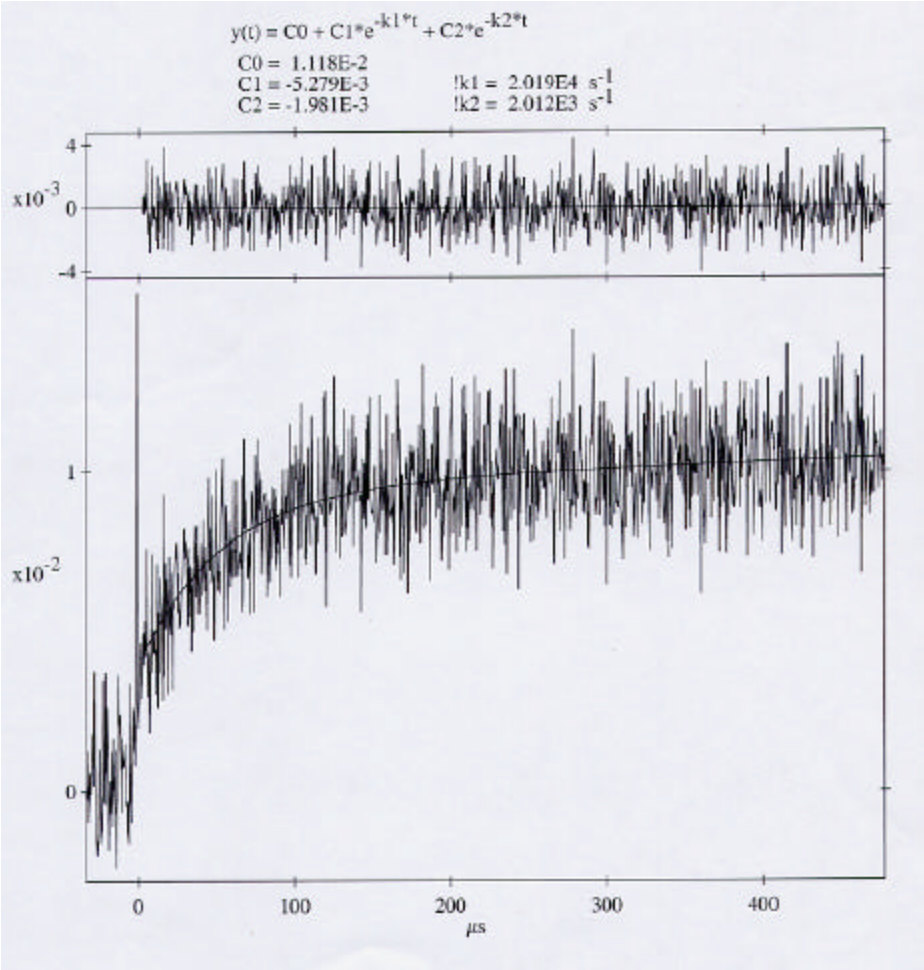
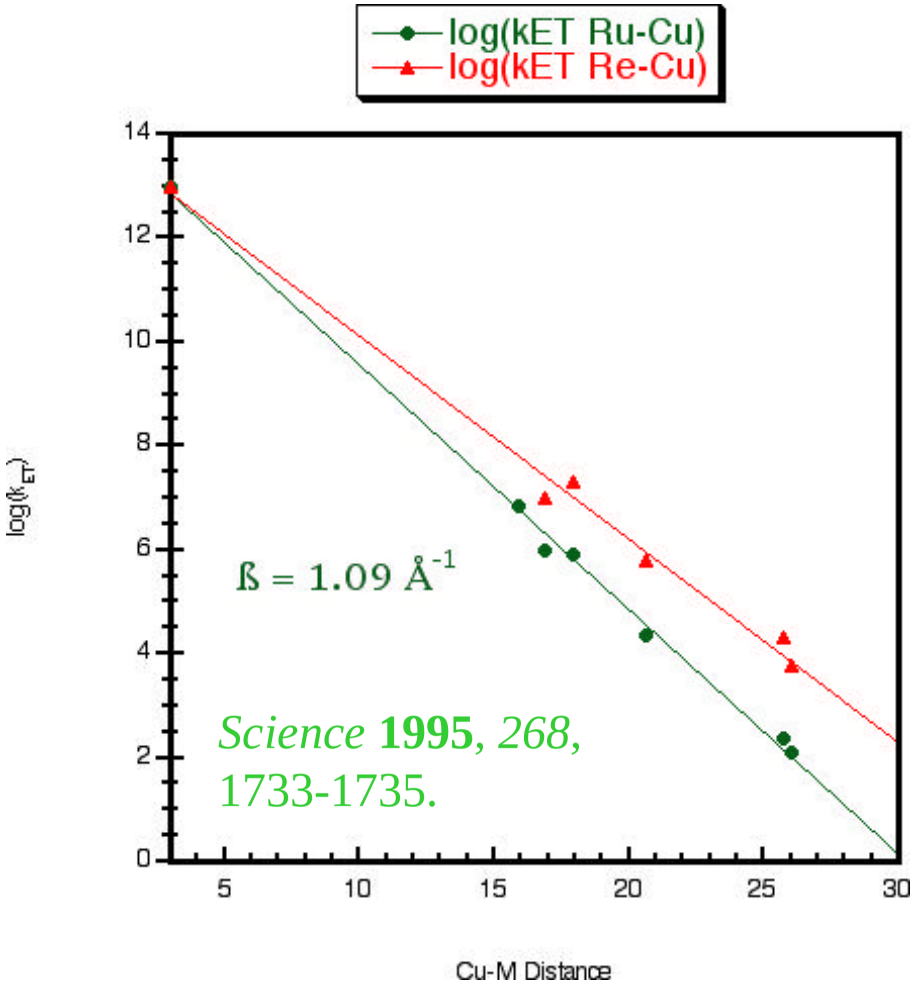

y(t) = C0 + C1*e^-k1*t + C2*e^-k2*t
C0 = 1.118E-2
C1 = -5.279E-3
C2 = -1.981E-3
!k1 = 2.019E4 s^-1
!k2 = 2.012E3 s^-1
x10^-3
4
0
-4
x10^-2
1
0
0
100
200
300
400
μs

Figure 6: Tunneling timetable for the oxidative flash quench rates: Ru v. Re.




*Science* **1995**, *268*, 1733-1735.

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*Biochim. Biophys. Acta*. **2003** (submitted)

**Figure 7**: X-band EPR spectrum of the oxidative flash quench product of the Re(His107) azurin taken at 77 K.

# Azurin Flash Quench

$$[Re(H107)]^{+}Az(F108)(XH)M^{2+} \xrightleftharpoons{h\nu} {}^{*}[Re(H107)]^{+}Az(F108)(XH)M^{2+}$$

$${}^{*}[Re(H107)]^{+}Az(F108)(XH)M^{2+} \xrightarrow{[Co(NH_3)_5Cl]^{2+}} [Re(H107)]^{2+}Az(F108)(XH)M^{2+}$$

$$[Re(H107)]^{2+}Az(F108)(XH)M^{2+} \xrightarrow[-H^{+}]{ET} [Re(H107)]^{+}Az(F108)(X\bullet)M^{2+}$$

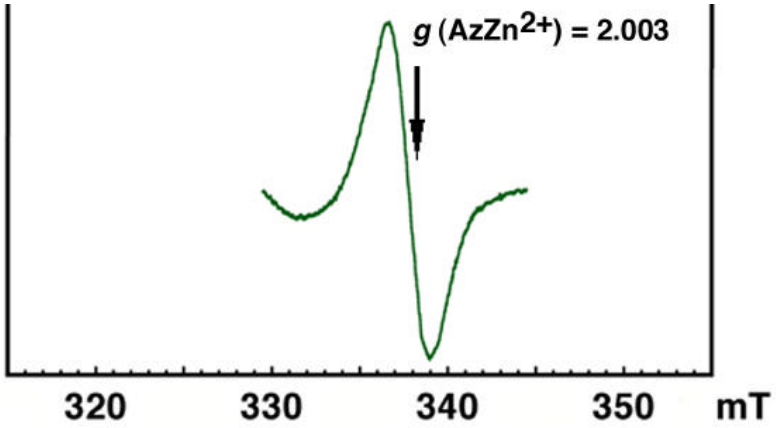


Figure 8: High-frequency EPR of Re(His107) photoproduct taken at 77K.

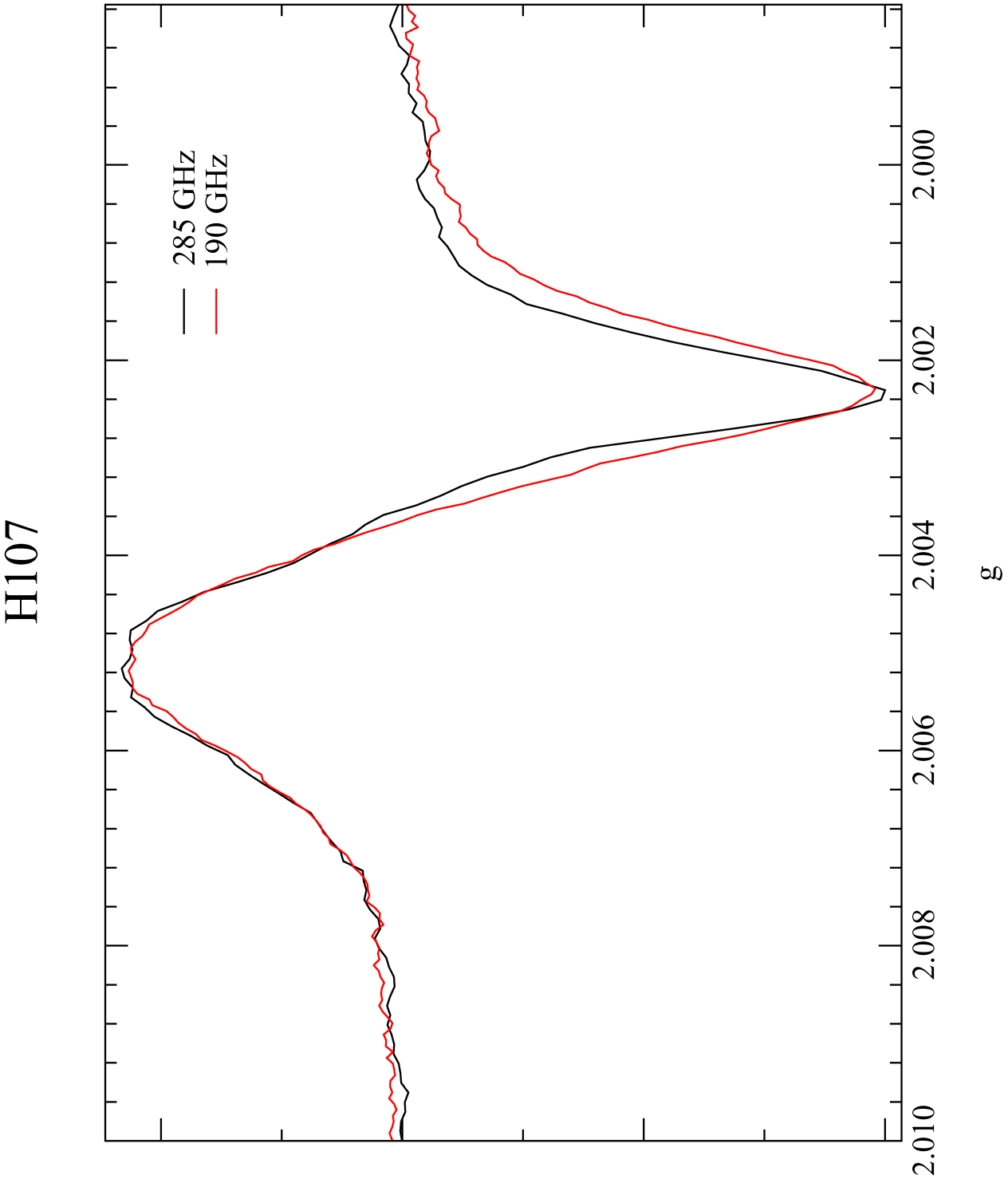
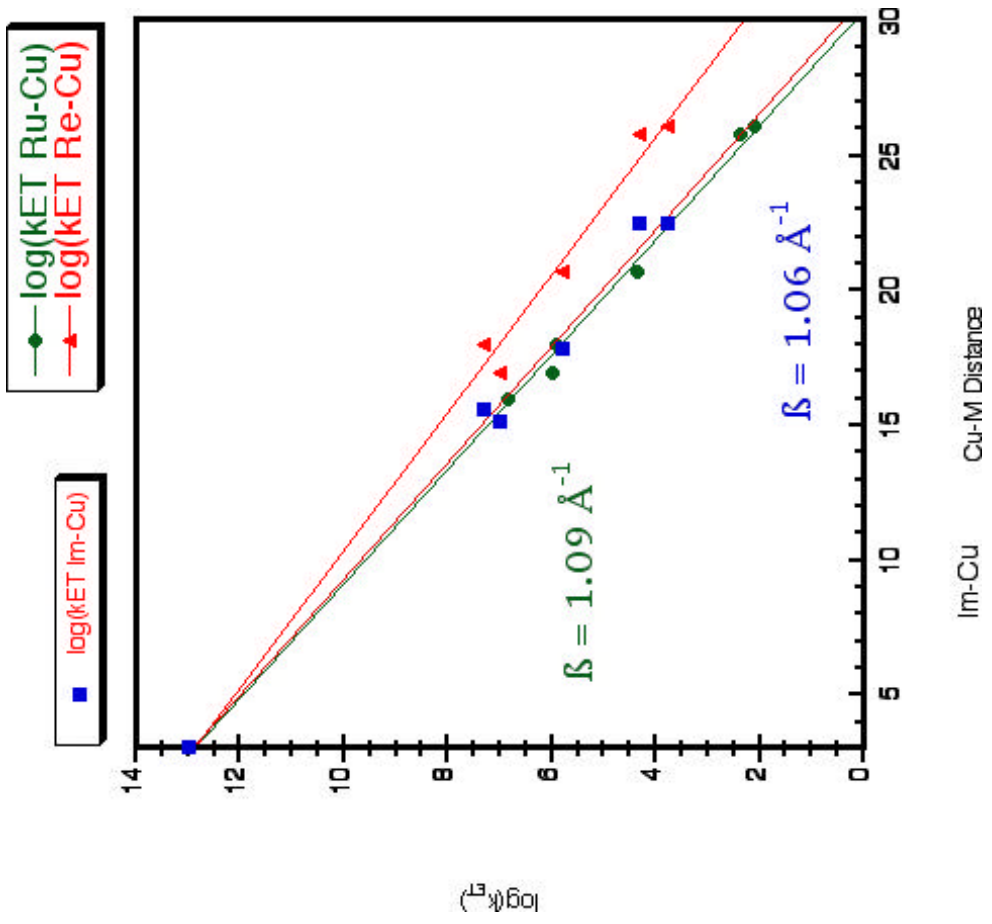

H107
285 GHz
190 GHz
2.010
2.008
2.006
2.004
2.002
2.000
g

Figure 9: Tunneling table of imidazole oxidations of Cu(I).


log(kET Ru-Cu)
log(kET Re-Cu)
log(kET Im-Cu)
ß = 1.09 Å-1
ß = 1.06 Å-1
log(kET)
Im-Cu Cu-M Distance
0
2
4
6
8
10
12
14
5
10
15
20
25
30

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# Chapter 3

## Tyrosyl Radicals

## Introduction

In the first experiments of electron transfer in rhenium modified azurins, the Re(His107) labeled protein showed an enhanced rate of electron tunneling over the analogous ruthenium derivative ($2x10^4$ $s^{-1}$ v. 200 $s^{-1}$). As the following residue is tyrosine 108, the initial model proposed that a tyrosyl radical intermediate is formed rapidly by reducing the $Re^{2+}$ species. This would then be followed by reduction of the tyrosyl radical by Cu(I), whose rate could be monitored by transient absorption kinetics of Cu(II) formation. The scheme for this proposal is shown in Figure 1.[1]

In this chapter we address the questions of whether the rhenium flash quench experiment can generate a tyrosyl radical at residue 108 and what role that radical may play in the multistep electron tunneling model. This system is well suited towards detailed studies of the spectroscopic properties of tyrosyl radicals. Furthermore, we have mutated the azurin protein in order to incorporate a tyrosine residue in a completely hydrophobic pocket (residue 48) and have undertaken the flash quench experiment from His83.

## Materials and Methods

The two mutants of azurin described in this chapter are (1) W48F/Y72F/H83Q/Q107H and (2) W48Y/Y72F/Y108F. The preparation of the rhenium labeled mutants, the photogeneration of the tyrosyl radicals, and the EPR detection of these radicals are described in the Appendices.

**Results**

Our initial experiments focused on the ability of the rhenium flash quench system to make the tyrosyl radical at residue 108. This was demonstrated in a zinc(II) derivative of the protein (negating any possibility of multi-step tunneling through oxidation of CuI). As the $g_{\perp}$ of blue copper[2] overlaps with EPR signal of the tyrosyl radical, the preference of $Zn^{2+}$ to $Cu^{2+}$ in the active site that cannot undergo oxidation was made necessary. The X-band EPR spectrum of the tyrosyl 108 radical is shown in Figure 2 and clearly identifies the radical as tyrosyl in nature, with an effective g = 2.0042, and the characteristic doublet spitting (due to the β-protons). The simulation is also shown in Figure 2. The $g_y$ and $g_z$ components are standard for all tyrosyl radicals observed to date. The $g_x$ component ($g_x$ = 2.008) is the parameter sensitive to hydrogen bonding. The crystal structure[3] indicates a hydrogen bond to the backbone amide of lysine 103; glutamate 106 is in the vicinity, but is not directed towards the tyrosine residue (Figure 3). There is hyperfine coupling to the protons that are ortho to the phenolic carbon (which is comparable to other tyrosyl radicals, indicating a similar spin distribution)[4-6] and also to the two beta protons. These hyperfine values to the two beta protons are inequivalent, and can be related to the dihedral angle that the phenol ring makes each of these protons.[7] Azurin also contains a tyrosine residue at position 72. While this residue is found more than 20 Å from the rhenium unit, we mutated it to phenylalanine to avoid amibiguity in our EPR experiment, even though we do not think that Tyr72 can be oxidized preferentially over Tyr108 from Re(His107).

Having demonstrated the formation of tyrosyl radicals in rhenium-azurin, we undertook the high-frequencey, high-field EPR of tyrosyl radical 108 in collaboration with Professor Sun Un at the CEA-Saclay. High frequency EPR (at 190 and 285 GHz in the case of our experiments) provides a very accurate measurement of g-tensors in organic radicals (although the information about hyperfine interactions is lost, as they are much smaller than the microwave quanta). The high-frequency EPR spectra for tyrosyl radical 108, at 190 and 285 GHz are shown in Figure 4.

The wild-type azurin contains a Trp (48) residue in completely hydrophobic pocket. We have mutated this residue to Tyr, labeled His83 with rhenium, and repeated the flash quench experiment to see if we can make the tyrosyl radical (the formation of the Trp radical and the difference in their EPR spectra, revealing their sensitivity to the protein microenvironment are reported in Jeremiah Miller's thesis[8]). Figure 5 shows the X-band EPR spectrum of this radical. The simulation of this spectrum (Figure 6) shows the expected $g_y$ and $g_z$ components of g-tensor and the increased $g_x$ value of 2.0137. The effective g of 2.0048 value appears coincident with that of the tyrosyl radical at position 108 (Figure 7). The higher value of $g_x$ tracks with the decreased amount of hydrogen bonding character to the phenolic oxygen atom. We suggest that this radical is deprotonated (as is the case for tyrosyl 108 radical) and we attribute protein dynamical motions in the seconds it takes to freeze the EPR sample with allowing the proton to escape from the hydrophobic pocket. The hyperfine parameters show similar coupling for the ortho protons, although the values for the coupling to the beta protons suggest a different dihedral angle as compared to the tyrosyl 108 radical. We are currently

undertaking the high-frequency, high-field EPR of tyrosyl 48 radical with Professor Sun Un to obtain an accurate assessment of the g-tensor.

We would like to mention an on-going collaboration with Professor Stenbjorn Styring and Dr. Ann Magnuson at Lund University (Lund, Sweden) on the time-resolved EPR characteristics of the tyrosyl 108 radical. In the Trp48Phe/Tyr72Phe/His83Gln/Gln107His mutant (Re is at His107 and Tyr108 is present) the Tyr108 radical lives for 10 minutes (data are not shown). In the Tyr72Phe/His83Gln/Gln107His mutant (containing Tyr108 and Trp48) the Tyr radical lives for 15 *seconds.* We are currently pursuing the electron transfer between Tyr108 and Trp48, starting with both radicals (the oxidation of Tyr108 by Trp48 has been investigated by Jeremiah Miller in his thesis). The rhenium-azurin system is ideal for studying electron transfer between tyrosine and tryptophan residues, for which there have been studies using pulse radiolytically generated radicals.[9,10]

**Discussion**

Tyrosyl radicals have been studied more extensively than any other amino acid radical in proteins and enzymes.[6] In this regard, they become test cases for the rhenium flash quench system. EPR studies of two tyrosyl radicals in different protein environments confirmed the rhombic ($g_x$ ? $g_y$ ? $g_z$) nature of the g-tensor. As the $g_x$ component is sensitive to the strength of the hydrogen bond to the radical, it provides an important experimental parameter for assessing the polarity and solvent accessibility of the protein microenvironment.[11-13] High-frequency EPR studies have been pursued on

tyrosyl radicals in RNR,[14,15] PSII,[16] and prostaglandin H synthase,[17] and provide accurate determinations of the g-tensor components. Our work on tyrosyl radicals complements the existing studies, and, in the case of the radical at Y48, provides a reference point for a tyrosyl radical in a completely hydrophobic environment. Simulations of the X-band EPR spectrum give a $g_x$ value of 2.0137, the highest $g_x$ value for tyrosyl radicals measured to date. The protein microenvironment around residue 48 when it is tyrosine provides the reference point for the electronic structure of tyrosyl radicals in a hydrophobic environment. High-frequency EPR experiments on the Tyr48 radical are currently in progress to obtain an accurate measure of this important parameter

The question remains as to the role of tyrosyl radicals in electron transfer processes in our rhenium-modified azurins. Electrochemical work has found the redox potential to be ~ 1 eV at pH 7.[18] This should provide sufficient driving force to oxidize Cu(I), which has an E° $Cu^{2+/+}$ ~ 300 meV.[2] Our experiments do not support Cu(I) oxidation by tyrosyl radicals as the rates of oxidation are the same in proteins containing tyrosine residues as they are in Tyr → Phe mutations (Chapter 2, this thesis). The current model for the rhenium-azurin systems has a rhenium-coordinated histidine radical forming after the quenching of the excited state. This is followed by oxidation of tyrosines or Cu(I). In the Re(His107) protein, this can imply that the rate of tyrosyl radical formation is less than the rate of Cu(I) oxidation ($2 \times 10^4$ $s^{-1}$), which means that tyrosyl radical formation is not kinetically favorable. However, pulse radiolytic studies on His-Tyr dipeptides suggest rapid tyrosyl radical formation following selective histidine oxidation ($k > 10^8$ $s^{-1}$).[19] With this in mind, tyrosyl radicals can still be formed as competing processes to Cu(I) oxidation. We are currently investigating the transient

absorption spectroscopy of the tyrosine mutants to determine the role of tyrosyl radicals during the Cu(I) oxidation event.

Figure 1: Proposed electron reaction scheme with a tyrosyl “hop.” (adapted from Winkler et al.)[1]

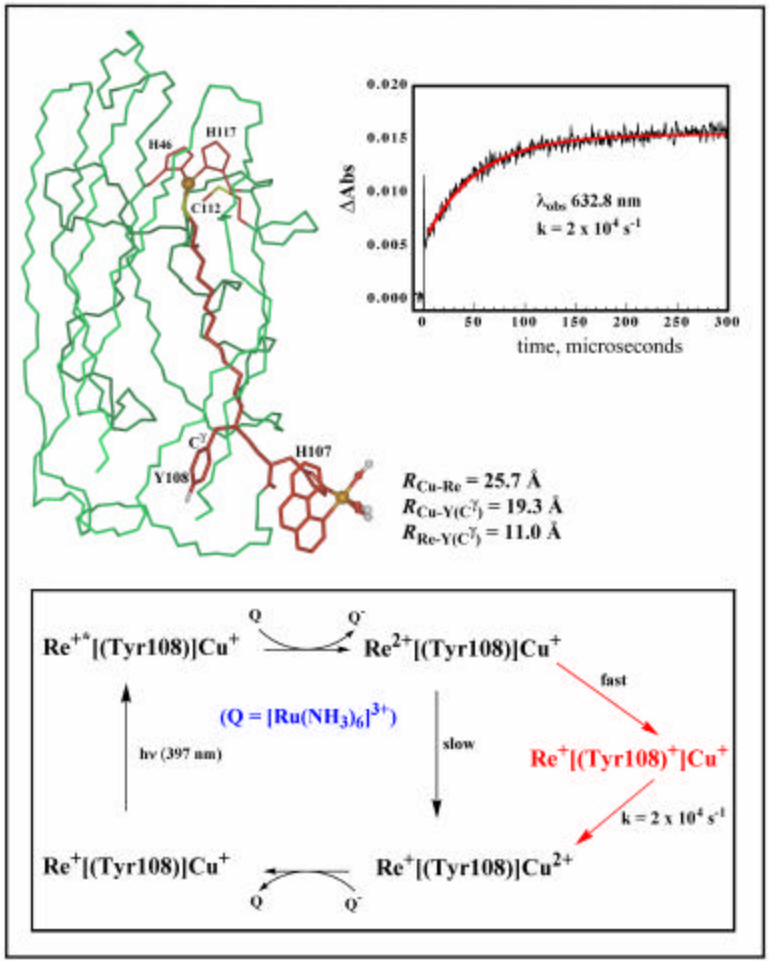
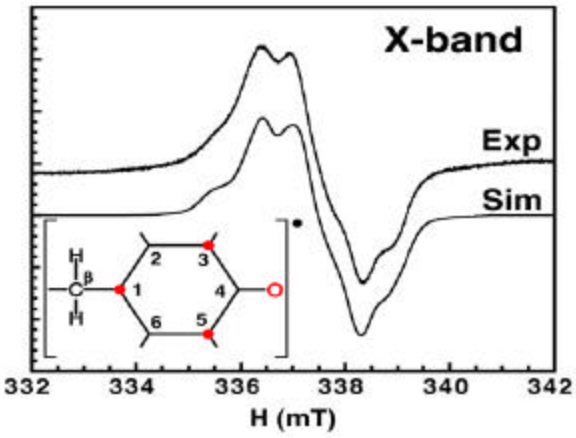

H46
H117
C112
Y108
H107
0.020
0.015
0.010
0.005
0.000
ΔAbs
λobs 632.8 nm
k = 2 x 10⁴ s⁻¹
0
50
100
150
200
250
300
time, microseconds
RCu-Re = 25.7 Å
RCu-Y(Cγ) = 19.3 Å
RRe-Y(Cγ) = 11.0 Å
Re+*|(Tyr108)|Cu+
Q
Q-
Re2+|(Tyr108)|Cu+
fast
(Q = [Ru(NH3)6]3+)
hν (397 nm)
slow
Re+|(Tyr108)+|Cu+
k = 2 x 10⁴ s⁻¹
Re+|(Tyr108)|Cu+
Re+|(Tyr108)|Cu2+
Q
Q-

Figure 2. Tyr108 radical EPR and EPR simulation




$g_x = 2.0080$
$g_y = 2.0040$
$g_z = 2.0018$

Figure 3:  Crystal structure showing the environment around Tyr108.

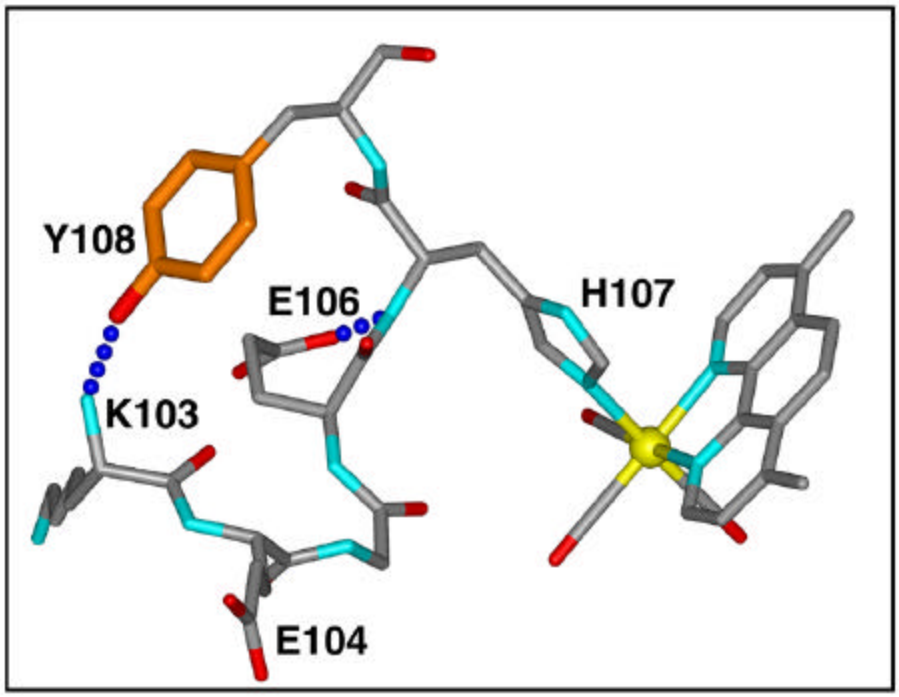

Y108
E106
H107
K103
E104

Figure 4. Tyr108 radical high field EPR

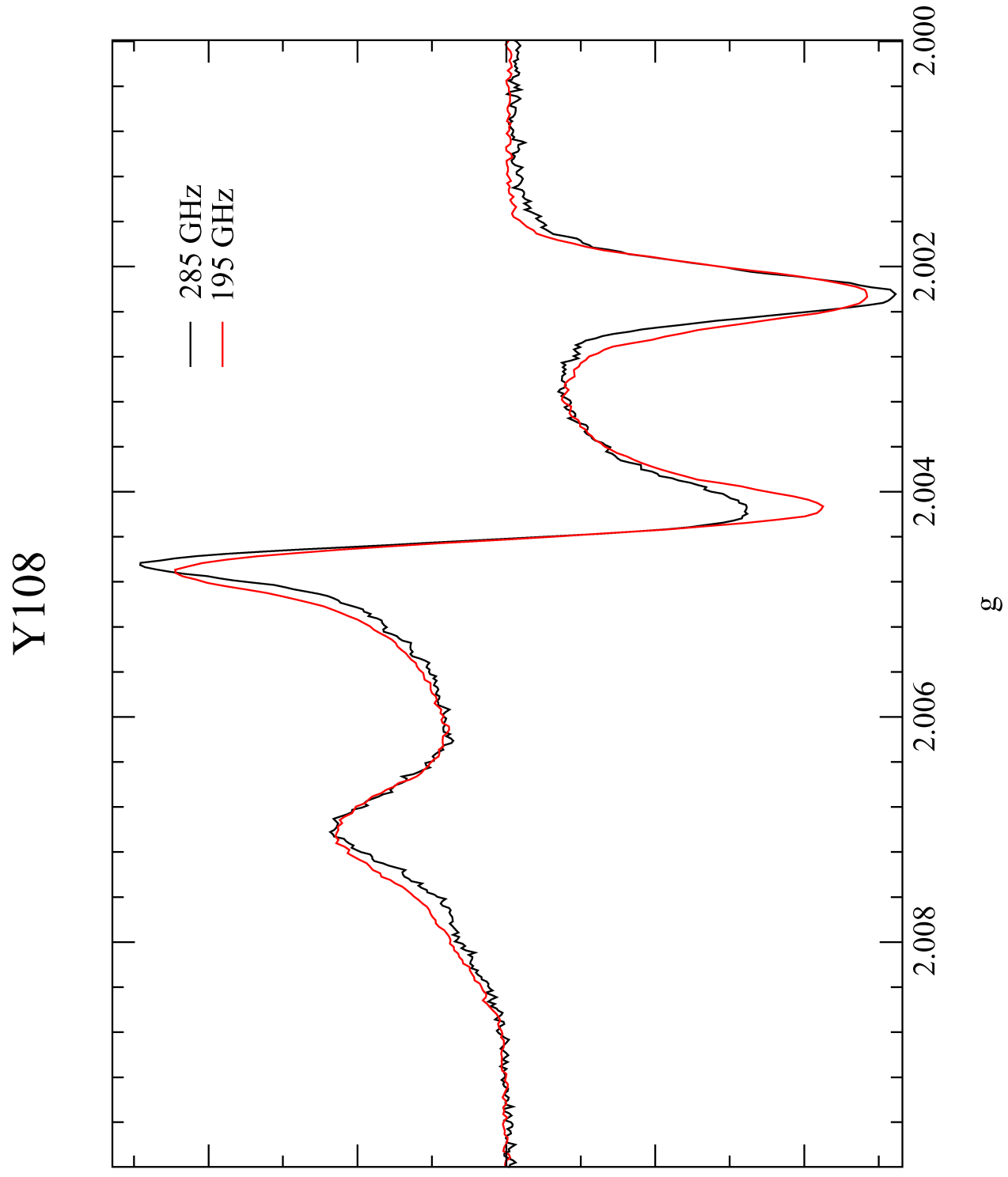
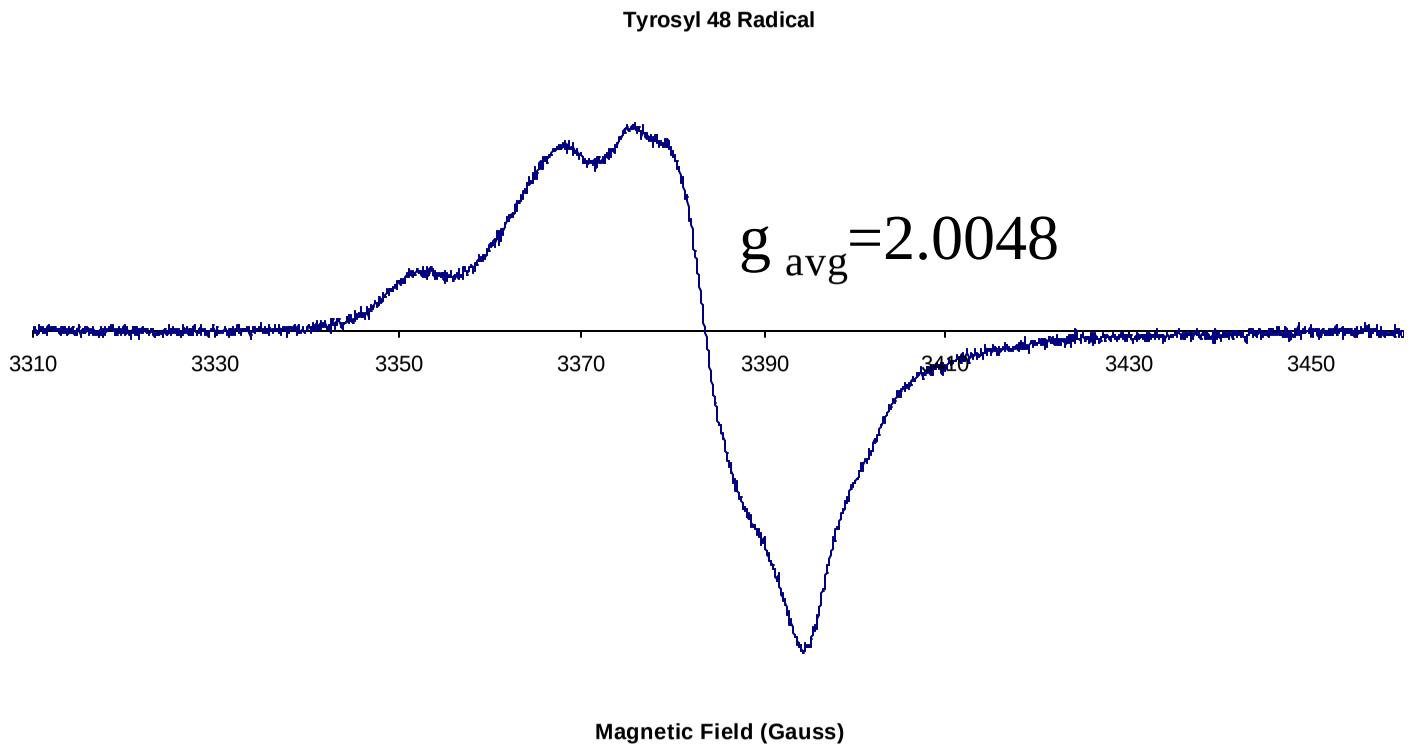

Y108
285 GHz
195 GHz
2.000
2.002
2.004
2.006
2.008
g

Figure 5. Tyr48 radical EPR




$g_x$=2.0137, $g_y$=2.0047, $g_z$=1.9968

Figure 6. Tyr48 radical simulations

The experimental spectrum appears in blue and the simulated spectrum appears in green.

Parameters:

$a_{1x}$=3 $a_{1y}$=3 $a_{1z}$=0.5 G

$a_{2x}$=3 $a_{2y}$=3 $a_{2z}$=3.5 G

$a_{3x}$=12 $a_{3y}$=4 $a_{3z}$=3 G

$a_{4x}$=15 $a_{4y}$=4 $a_{4z}$=0.5 G

$g_x$=2.0137

$g_y$=2.0047

$g_z$=1.9968

linewidth L/G =0

$s_x$ 3.5 $s_y$=3.5 $s_z$=3.0

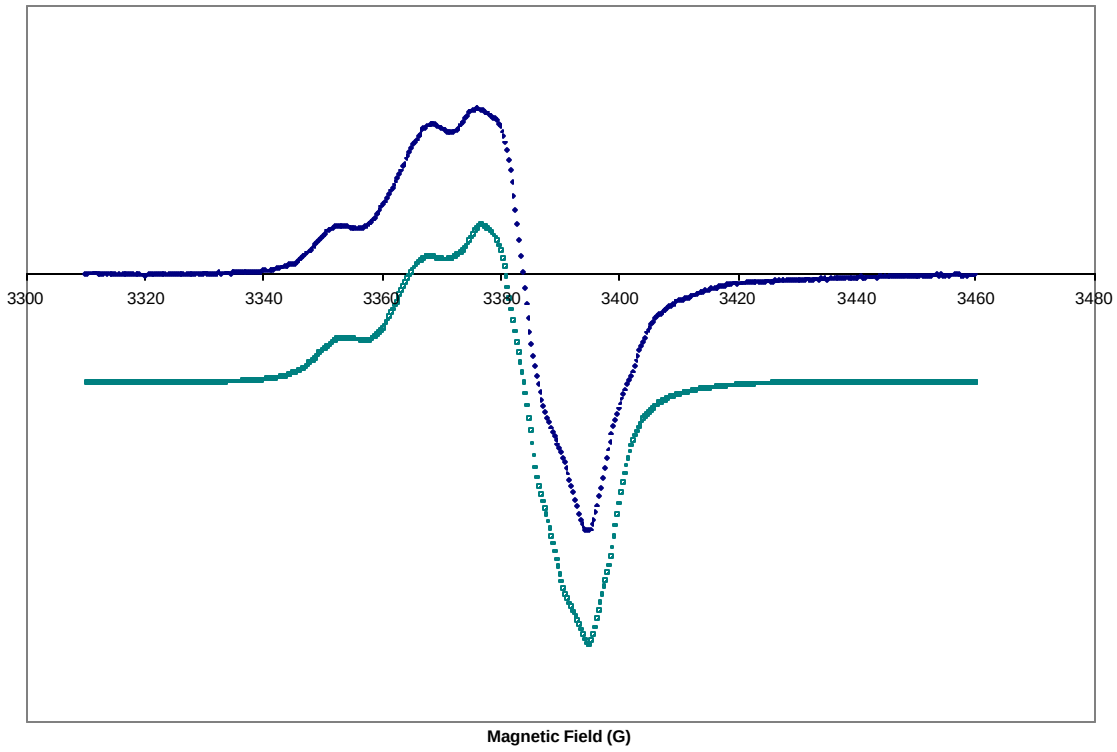
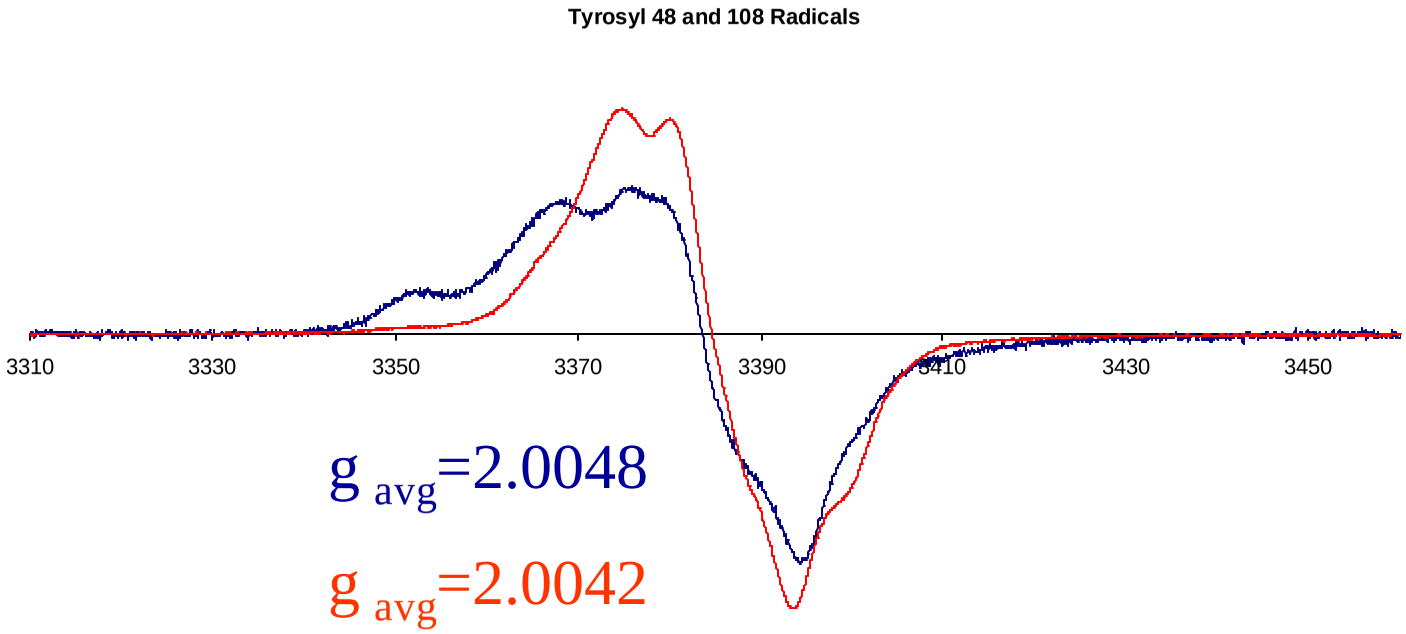

3300
3320
3340
3360
3380
3400
3420
3440
3460
3480
Magnetic Field (G)

Figure 7:  Overlay of Tyr48 and Tyr108 radicals




$g_{avg}$=2.0048

$g_{avg}$=2.0042

$g_x$=2.0137, $g_y$=2.0047, $g_z$=1.9968

$g_x$=2.0080, $g_y$=2.0040, $g_z$=2.0018

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# Chapter 4

## Cysteine-Based Radicals

## Introduction

The oxidative flash quench experiments of $[Re(CO)_3(phen)(His)]^+$ complexes in various mutants of *Pseudomonas aeruginosa* azurin have demonstrated the formation of tyrosyl and tryptophan radicals. Another major class of amino acid radicals that could be studied by the rhenium flash quench method in azurin is based on cysteine. Considering that cysteine thiyl radicals are postulated to initiate ribonucleotide reduction in RNR and are the catalytically active component in all glycyl radical enzymes, the study of their properties and reactivities in model proteins would provide great insight into their roles in enzyme catalysis.

The study of radicals derived from thiols dates to the 1960s and 1970s, when Symons[1-3] and others[4-6] investigated the EPR spectra of irradiated solutions and crystals of sulfur compounds, including L-cysteine. Multiple products were detected, including disulfide radical cations and anions, the perthiyl radical, and various oxygen derivatives of the thiyl radical (Figure 1). Symons presented arguments on the difficulty (though not impossibility) to observe a pure thiyl radical signal by EPR (its reactivity notwithstanding) due to the near degeneracy of the $p_x$ and $p_y$ orbitals in which the unpaired electron resides. With an orbitally degenerate ground state and the larger value of the spin-orbit coupling constant for sulfur compared to carbon, the signal would be expected to be inhomogenously broadened, which is the case compared to carbon-based radicals. In addition, thiyl radical assignments could not be made based on g values alone because of two complicating factors: hydrogen bonding to the thiyl radical breaks the orbital degeneracy and leads to a shift in the $g_1$; and in the pulse radiolysis

experiments, multiple sulfur radical products are produced (not selectively) that have EPR parameters that are remarkably similar to one another. The $g_1$ component of the axial g-tensor is diagnostic of a thiyl radical as all other sulfur-centered radicals have rhombic g-tensors.

In this chapter, we address the formation and properties of cysteine based radicals in azurin. EPR studies on photogenerated species have been complimented with density functional theory calculations on thiyl radicals to provide a quantitative handle on the electronic structures of thiyl radicals and the effects of the electronic structures on EPR spectra. In particular, the $g_1$ value of the g-tensor is monitored as a function of the energy separation between the $p_x$ and $p_y$ orbitals (and hence the strength of the hydrogen bond to the radical) and the effective spin-orbit coupling parameter, a direct measure of covalency to the β methylene group. Alkoxyl and selenyl radicals, which have smaller and larger spin-orbital coupling values than thiyl radicals, have also been subjected to the same computations and analyses. Our calculations are the first since 1966[7] when sulfur and selenium based radicals were treated by extended Huckel method of molecular orbitals as described by Ballhausen and Gray.[8] Although Engstrom has recently calculated EPR parameters of sulfur centered radicals, no understanding in terms of molecular orbitals is provided.

**Materials and Methods**

The two mutants of azurin described in this chapter are (1) W48F/Y72F/H83Q/Q107H/Y108C and (2) W48C/Y72F/Y108F. The preparation of the rhenium labeled mutants, the

photogeneration of the cysteine-based radicals, and the EPR detection of these radicals are described in the Appendices.

**Results and Discussion**

The EPR spectrum of the oxidative flash quench product of the His107 (Tyr108Cys) rhenium labeled protein is shown in Figure 2. The isotropic g-value (2.0104) is found to be greater than the free electron g-value and the g-values of the histidinyl and tyrosyl radicals presented in Chapters 2 and 3 of this thesis, respectively. This is indicative of a sulfur based radical species as the larger spin-orbit coupling value of sulfur versus oxygen or carbon causes a shift towards greater g-values.

The spectrum does not have an axial g-tensor as would be expected for a thiyl radical; the resolved hyperfine coupling cannot be explained in terms of a thiyl radical either. Based on a similar EPR spectrum of a radical species generated in RNR by Graslund (Figure 3),[9] we assign this species to a sulfinyl radical (RSO·). The simulation of the spectrum g-tensor components of $g_1 = 2.0202$, $g_2 = 2.0118$, $g_3 = 1.9992$. These parameters are almost identical to Graslund sulfinyl radical in RNR and Sevilla's sulfinyl radical in pulse radiolytic studies.[10-15] The resolvable hyperfine splitting is due to one beta proton.

The formation of a sulfinyl radical on cysteine 108 was a surprising result. Mass spectrometry on the protein (mutant W48F/Y72F/H83Q/Q107H/Y108C), labeled and unlabled with rhenium, gave the expected masses of 13828 and 14278 (M + 450)

respectively, as expected. Thus, cysteine 108 was in the thiol(ate) oxidation state. We had considered that the rhenium complex could have sensitized triplet oxygen to singlet oxygen, the latter of which is known to oxidize thiols to sulfenic and sulfinic acids. Satisfied that no post-translational reaction with oxygen had occurred and that the cysteine was in the thiol oxidation state, alternate mechanisms of sulfinyl radical formation had to be considered. One possible mechanism is presented in Figure 4. The scheme requires atmospheric oxygen to react with a thiyl radical to give the thiyl peroxyl species. Sevilla, in his studies on sulfur based radicals and their oxygen adducts, found that an increased $^{17}O$ hyperfine parameter ($A_{\|}$) on the terminal oxygen atom tracked with increased hydrogen abstracting ability, with the sequence and A|| values shown in Figure 5. We suggest that following initial formation of the thiyl peroxyl radical, this species abstracts a hydrogen atom from the protein to give the thiyl hydroperoxide (alternatively, the thiyl peroxyl radical can photoisomerize (with visible light) to the sulfonyl radical, but there is no evidence to indicate its formation in our experiments). This species undergoes homolytic cleavage of the O—O bond to give the product sulfinyl radical and hydroxyl radical. The fate of the hydroxyl radical in this mechanism is unknown. We suggest that the hydroxyl radical could diffuse to another azurin molecule where it can react with a thiyl radical to for a sulfenic acid. While sulfenic acids are rare and unstable, they have been found in several enzyme systems (and NADH peroxidase mutants)[16-18] and are postulated to be intermediates in redox signaling. A second flash quench would oxidize the sulfenic acid to the sulfinyl radical, although there is no independent confirmation or precedent for this one electron chemistry.

Whence oxygen? All samples were illuminated under an argon atmosphere (supposedly). However, the sulfinyl radical was also formed in samples irradiated under atmospheric oxygen and nitrogen pressures (and thus not degassed with argon). More rigorous means of oxygen removal are needed in order to have the chance to trap the thiyl radical at Cys108.

The sulfinyl radical was found to live for at least 10 minutes. In the freeze flash-quench experiment, the sample is frozen immediately after illumination (sample freezing time is estimated to be a few seconds). Upon thawing for 2 minutes, the sulfinyl spectrum remained, although the signal intensity had decreased but had become sharper. We are currently exploring whether there could have been an additional species (the thiyl radical itself) overlapping with the sulfinyl radical). Further thawing of the sample for 10 minutes followed by freezing revealed a similar EPR spectrum.

The EPR spectrum for the irradiated His83 Cys48 protein is shown in Figure 5. The resonating feature cannot be conclusively assigned. It appears where the $g_{\perp}$ of thiyl radicals are found and where the $g_{\parallel}$ of blue copper is found. As samples had a blue tint, there was a blue copper impurity whose $g_{\parallel}$ masked the diagnostic $g_{\parallel}$ of the thiyl radical. We are still pursuing this thiyl radical by EPR in zinc and apo preparations of rhenium-modified azurin.

A preliminary x-ray absorption (XAS) experiment by Dr. Pierre Kennepohl (personal communication) has demonstrated the 1s $\rightarrow$ 3p transition in the oxidative flash quench product of His83 Cys48, suggesting thiyl radical formation and necessitating further investigation by EPR.

In order to understand the electronic structure of thiyl radicals and the orbital molecular orbital basis for their EPR parameters, DFT calculations were carried out. We were especially interested in a quantitative understanding of the effects of hydrogen bond strength on the energy separation between the $p_x$ and $p_y$ orbitals and its effects on the $g_{|}$ value. Our study provides the details to understand what Symons qualitatively (and correctly) proposed in the 1970s on the relation between hydrogen bond strength, the $p_x - p_y$ energy separation, and the $g_{|}$ component of the g-tensor. The two extreme structural types are the deprotonated thiyl radical, RS·, and the thiyl radical cation RSH·+, where R = $CH_3CH_2$ in the calculations. Hydrogen bonding at various strengths were modeled by including $H_3O^+$, $H_2O$, and $OH^-$ in calculations on the neutral thiyl radical. Species are considered to be in the gas and aqueous phases, with solvation taken into account in these calculations by using the COSMO continuum dielectric model. A correlation diagram of the $p_x$, $p_y$, and $p_z$ orbitals for the gas phase calculations is shown in Figure 6. Even with the absence of a hydrogen bond ($CH_3CH_2S$·) the energy separation between $p_x$ and $p_y$ is found to be 3600 $cm^{-1}$. This separation increases to 22000 $cm^{-1}$ for the thiyl radical cation (both of these were taken from the gas phase calculations). It should be noted at this point that in the thiyl radical cation, the $p_z$ orbital now exceeds the energy of the $p_y$ orbital. We have undertaken similar calculations for the alkoxyl and selenyl radicals and have found the orbital picture to be qualitatively similar to that of the thiyl radical.

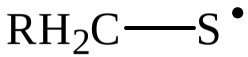
EPR parameters based on this MO picture are plotted in Figure 7. This plot includes the results of the calculation on alkoxyl, thiyl, and selenyl radicals, in the gas and aqueous phases. A linear correlation is found between the first order perturbative expansion term to the g-value equation ($2k\zeta/\Delta E$). $\Delta E$ is the energy difference between

the $p_x$ and $p_y$ orbitals; $\zeta$ is the spin-orbit coupling parameter; and k is the orbital reduction parameter, which accounts for radical character on the oxygen, sulfur, or selenium atom (as such it can provide a measure of covalency with the β-methylene). These results are compiled in Table 1.

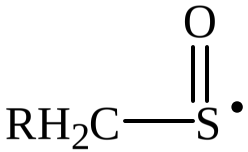
In Figure 8, we provide the quantitative basis for hydrogen bonding effects on the $p_x$ - $p_y$ energy separation and thus $g_{||}$ values of alkoxyl, thiyl, and selenyl radicals. This correlation is identical to what Symons set forth in the 1970s. Thus, the smallest energy separation, which is due to the non-hydrogen bonded neutral radical, gives rise to the largest $g_{||}$. The energies follow the trend O > S > Se, which means that the $g_{||}$ values would be expected to follow (and they do follow) the trend Se > S > O.

Figure 1. Some thiol derived sulfur based radicals

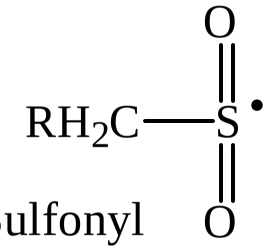
# Cysteine *Based* Radicals



Thiyl



Sulfinyl



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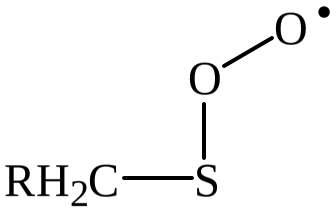
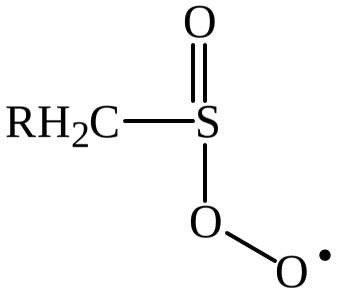


Thiylperoxyl



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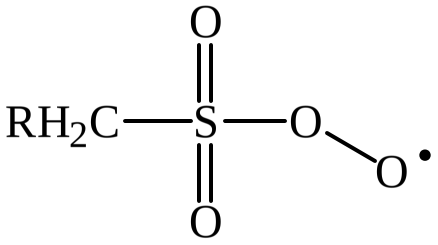
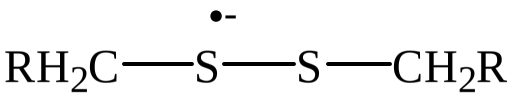
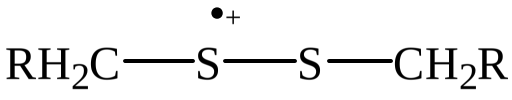


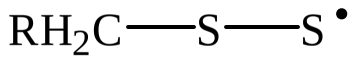
Sulfonylperoxyl



Disulfide radical anion

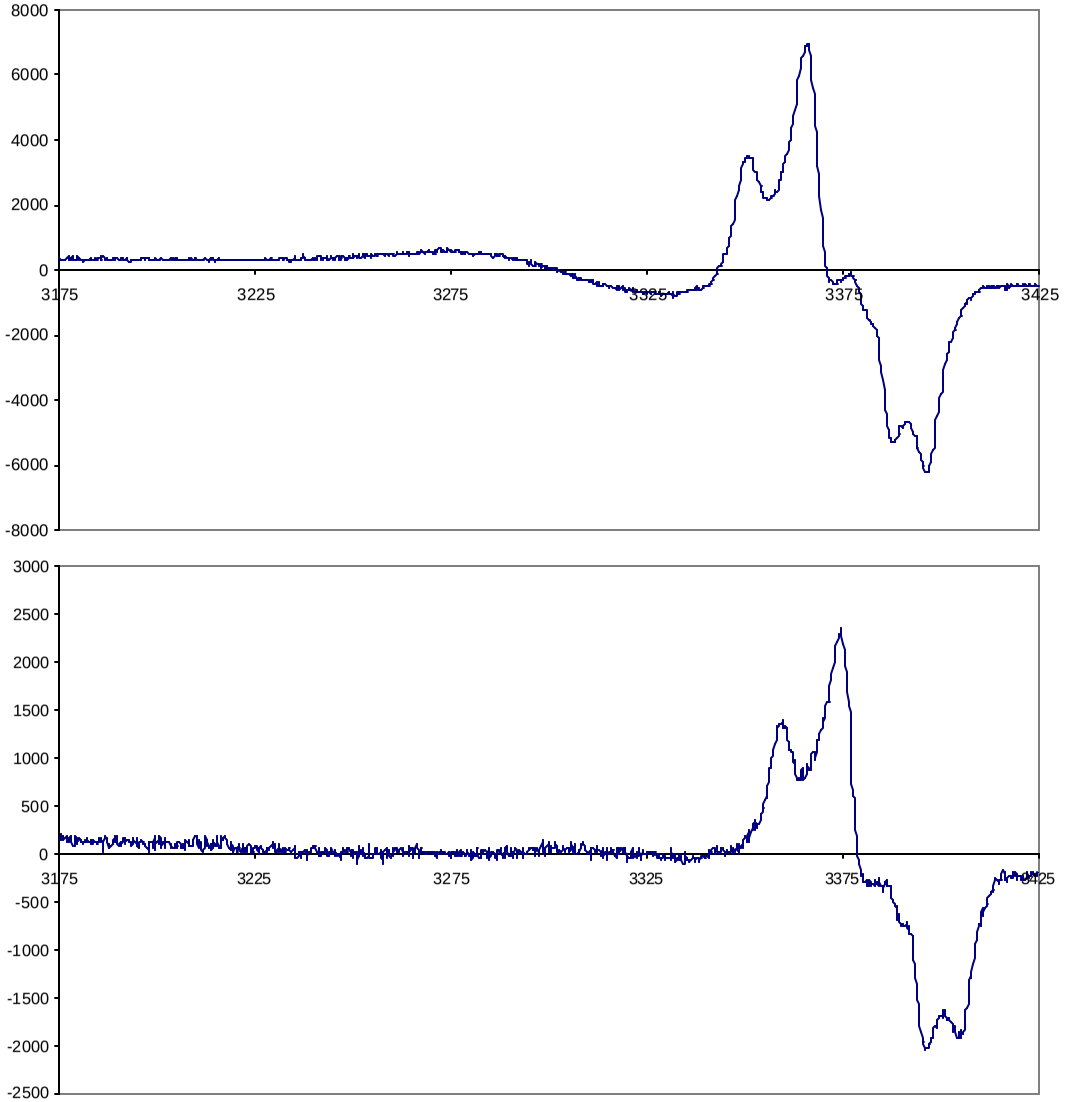


Disulfide radical cation



Perthiyl Radical

Figure 2.  Cys 108 EPR




t = 2 min, 10 min

$g_x = 2.0202$

$g_y = 2.0118$

$g_z = 1.9992$

Figure 3. Sulfinyl radical in RNR by Graslund *et al.*[9]

g tensor of RSO• inRNR

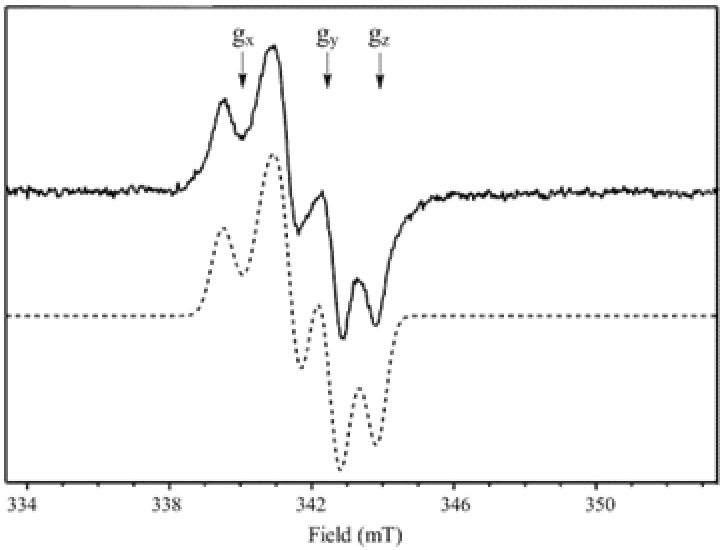
$g_x$= 2.0206

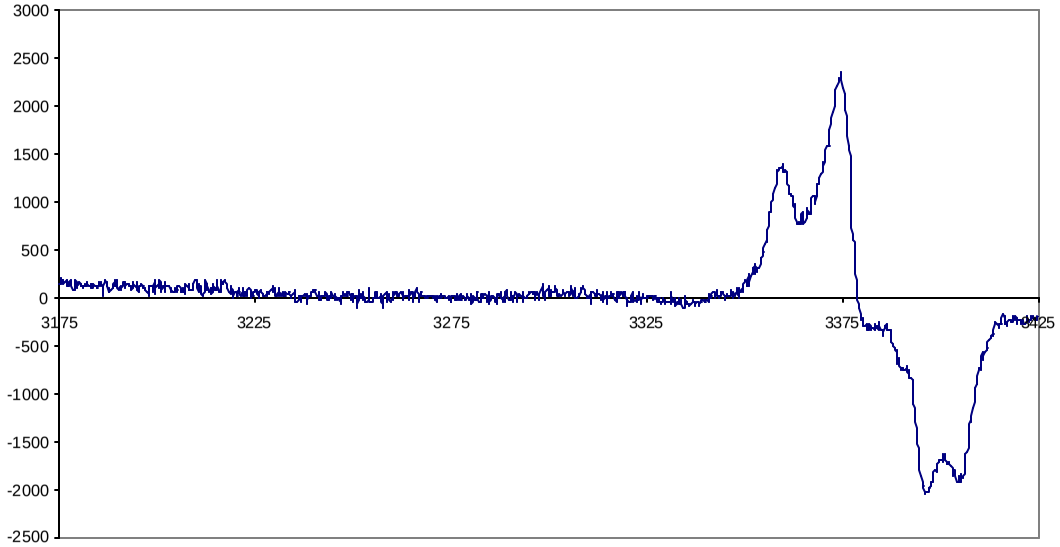
$g_y$= 2.0093

$g_z$= 2.0022

Gräslund et al.

*Biochemistry*. **2002**, *41*, 6510-6




t = 2 min, 10 min

$g_x$= 2.0202

$g_y$= 2.0118

$g_z$= 1.9992

Figure 4. Possible mechanisms of formation of the sulfinyl radical

Reaction Mechanism for the formation of RSO·

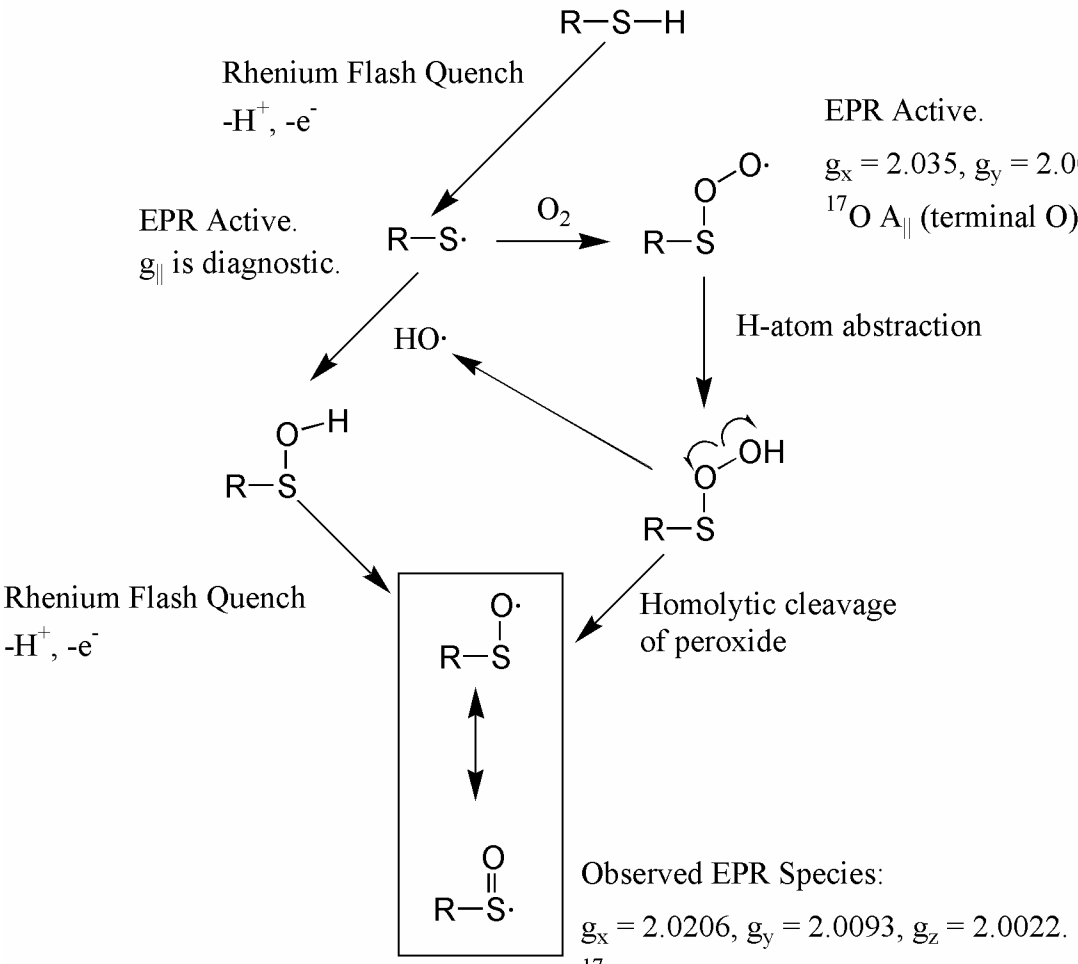


Figure 5. Cys48 EPR

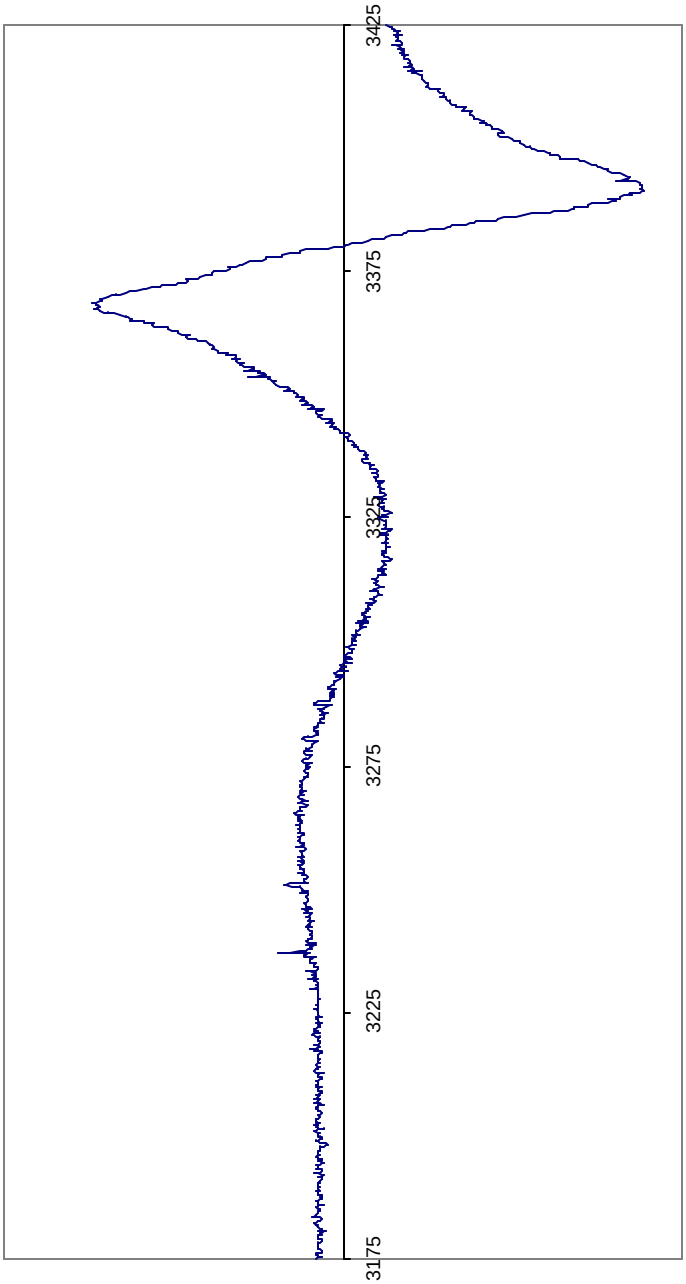

3425
3375
3325
3275
3225
3175

Figure 6. Theoretical calculations of the $g_x$ dependence on hydrogen bonding versus the energy separation between $p_x$ and $p_y$ in thiyl radicals.

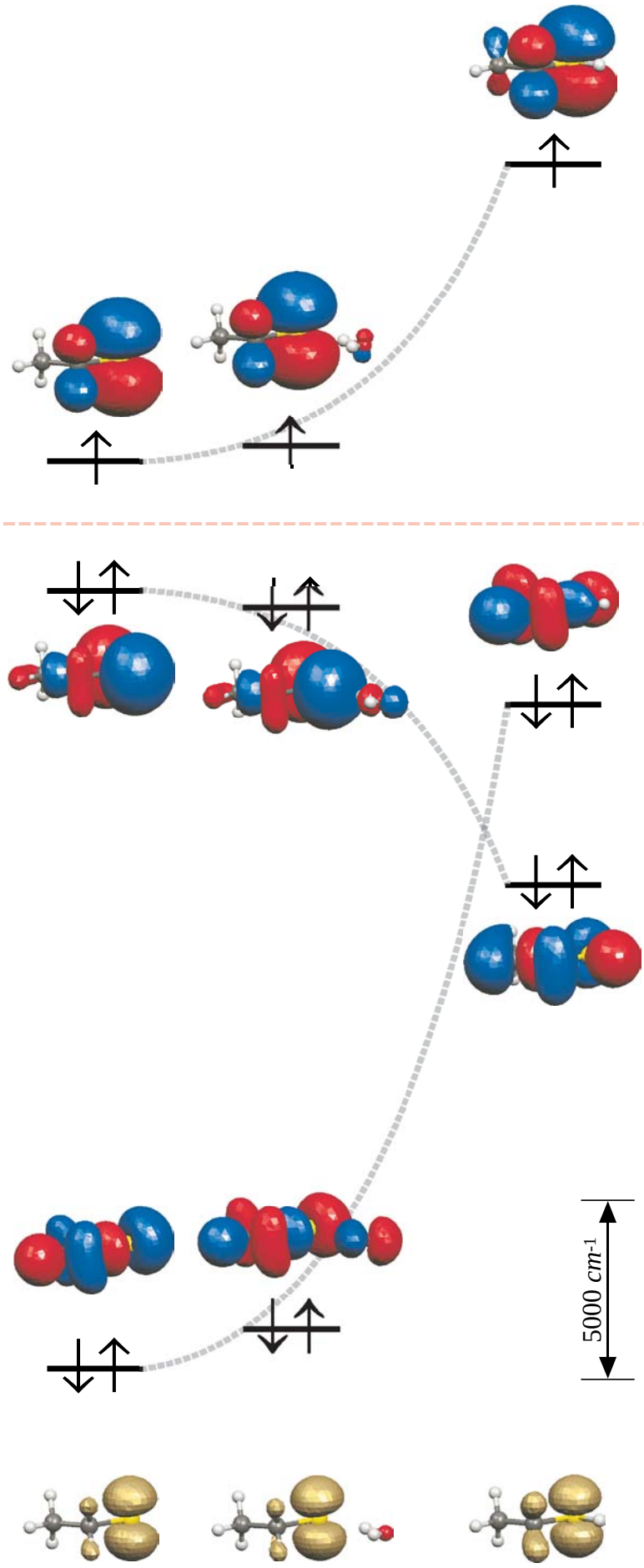

5000 $cm^{-1}$

Figure 7: Plot of the dependence between energy separation and the shift in $g_1$ value.

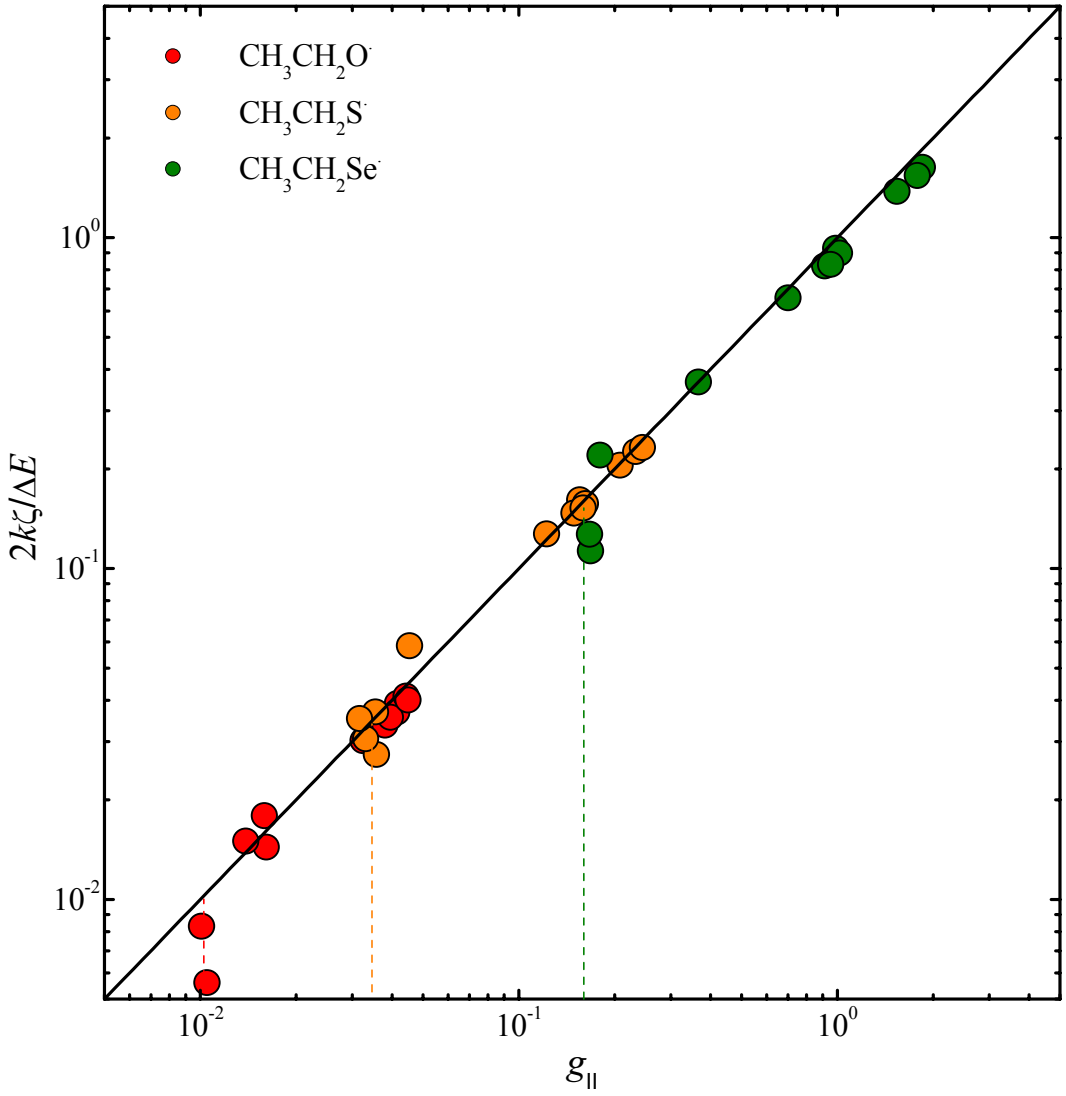

CH3CH2O
CH3CH2S
CH3CH2Se
10^0
10^-1
10^-2
2kζ/ΔE
10^-2
10^-1
10^0
g||

Table 1: Compilation of molecular orbital and EPR parameters of alkoxyl, thiyl, and selenyl radicals.

| | $\Delta g_3$ | $\Delta g_2$ | $\Delta g_1$ | $\Delta g_{avg}$ | $S_E$ | Ep1 | Ep2 | $\Delta g_1$ | $2k\zeta/\Delta E$ |
|---|---|---|---|---|---|---|---|---|---|
| $[ROH^{\cdot}]^+$ *(aq)* | -175.8 | 4415.5 | 10518.9 | 4919.555 | 0.4425 | -10.1280 | -7.1840 | 0.0105 | 0.0056 |
| $[ROH^{\cdot}]^+$ *(g)* | -169.1 | 4271.3 | 10098.8 | 4733.650 | 0.4535 | -15.3311 | -13.3044 | 0.0101 | 0.0083 |
| $[RO^{\cdot}][H_3O]^+$ *(aq)* | -208.8 | 6008.0 | 16059.5 | 7286.229 | 0.5702 | -8.1380 | -6.6680 | 0.0161 | 0.0144 |
| $[RO^{\cdot}][H_3O]^+$ *(g)* | -177.6 | 5287.4 | 13875.3 | 6328.389 | 0.5616 | -13.4648 | -12.0737 | 0.0139 | 0.0150 |
| $[RO^{\cdot}][H_2O]$ *(aq)* | -185.1 | 6839.0 | 32383.4 | 13012.421 | 0.7384 | -6.3880 | -5.4800 | 0.0324 | 0.0302 |
| $[RO^{\cdot}][H_2O]$ *(g)* | -161.6 | 6964.7 | 34363.5 | 13722.219 | 0.7878 | -6.5210 | -5.6810 | 0.0344 | 0.0349 |
| $[RO^{\cdot}][HO]^-$ *(aq)2* | 1431.9 | 13530.0 | 15864.8 | 10275.591 | 0.5142 | -5.1440 | -4.0730 | 0.0159 | 0.0179 |
| $RO^{\cdot}$ *(aq)* | -202.6 | 6894.1 | 38016.1 | 14902.528 | 0.7563 | -6.0620 | -5.2250 | 0.0380 | 0.0336 |
| $RO^{\cdot}$ *(g)* | -191.9 | 6909.3 | 41613.1 | 16110.200 | 0.8144 | -5.9251 | -5.1511 | 0.0416 | 0.0391 |
| $CH_3O^{\cdot}$ *(aq)* | -208.3 | 7082.6 | 41428.3 | 16100.859 | 0.7941 | -6.1140 | -5.3110 | 0.0414 | 0.0368 |
| $CH_3O^{\cdot}$ *(g)* | -200.2 | 7046.6 | 44334.7 | 17060.367 | 0.8387 | -6.0430 | -5.2840 | 0.0443 | 0.0411 |
| $HO^{\cdot}$ *(aq)* | -214.6 | 6973.4 | 39482.5 | 15413.770 | 1.0085 | -7.4010 | -6.3440 | 0.0395 | 0.0355 |
| $HO^{\cdot}$ *(g)* | -209.7 | 6693.1 | 44931.0 | 17138.126 | 1.0059 | -7.4320 | -6.4980 | 0.0449 | 0.0401 |
| $[RSH^{\cdot}]^+$ *(aq)* | 255.9 | 16405.5 | 35695.2 | 17452.223 | 0.9002 | -9.6101 | -6.4332 | 0.0357 | 0.0274 |
| $[RSH^{\cdot}]^+$ *(g)* | -26.2 | 12638.0 | 33016.3 | 15209.367 | 0.8628 | -15.1187 | -12.3918 | 0.0330 | 0.0306 |
| $[RS^{\cdot}][H_3O]^+$ *(aq)* | 185.9 | 23975.4 | 45423.7 | 23195.003 | 0.9308 | -7.3940 | -5.8520 | 0.0454 | 0.0584 |
| $[RS^{\cdot}][H_3O]^+$ *(g)* | -24.3 | 18260.1 | 35460.3 | 17898.724 | 0.8993 | -13.6769 | -11.3194 | 0.0355 | 0.0369 |
| $[RS^{\cdot}][H_2O]$ *(aq)* | -24.1 | 20760.4 | 122098.9 | 47611.739 | 0.9442 | -5.7080 | -4.9900 | 0.1221 | 0.1272 |
| $[RS^{\cdot}][H_2O]$ *(g)* | -29.1 | 19711.9 | 155101.4 | 58261.387 | 0.9445 | -5.9180 | -5.3520 | 0.1551 | 0.1614 |
| $[RS^{\cdot}][HO]^-$ *(aq)2* | -123.7 | 12017.2 | 31610.4 | 14501.309 | 0.6232 | -4.7430 | -3.0310 | 0.0316 | 0.0352 |
| $RS^{\cdot}$ *(aq)* | -65.1 | 20308.8 | 148926.6 | 56390.120 | 0.9424 | -5.4769 | -4.8575 | 0.1489 | 0.1471 |
| $RS^{\cdot}$ *(g)* | -43.9 | 19039.0 | 207873.6 | 75622.911 | 0.9484 | -5.4029 | -4.9560 | 0.2079 | 0.2052 |
| $CH_3S^{\cdot}$ *(aq)* | -93.0 | 19844.9 | 161655.9 | 60469.244 | 0.9479 | -5.4530 | -4.8700 | 0.1617 | 0.1572 |
| $CH_3S^{\cdot}$ *(g)* | -80.4 | 18449.1 | 232752.3 | 83707.019 | 0.9549 | -5.4220 | -5.0130 | 0.2328 | 0.2258 |
| $HS^{\cdot}$ *(aq)* | -150.8 | 17898.1 | 159051.6 | 58932.970 | 1.0130 | -6.0540 | -5.4110 | 0.1591 | 0.1524 |
| $HS^{\cdot}$ *(g)* | -152.2 | 16574.7 | 244611.7 | 87011.381 | 1.0105 | -6.2450 | -5.8240 | 0.2446 | 0.2321 |
| $[RSeH^{\cdot}]^+$ *(aq)* | 515.4 | 78547.0 | 167785.7 | 82282.720 | 0.9457 | -9.4162 | -6.0996 | 0.1678 | 0.1131 |
| $[RSeH^{\cdot}]^+$ *(g)* | 909.0 | 62208.1 | 166564.7 | 76560.612 | 0.9223 | -14.8495 | -11.9652 | 0.1666 | 0.1269 |
| $[RSe^{\cdot}][H_3O]^+$ *(aq)* | 533.4 | 115616.8 | 366395.6 | 160848.591 | 0.9643 | -6.2420 | -5.1970 | 0.3664 | 0.3661 |
| $[RSe^{\cdot}][H_3O]^+$ *(g)* | 943.5 | 102128.2 | 179725.3 | 94265.690 | 0.9433 | -12.5410 | -10.8430 | 0.1797 | 0.2204 |
| $[RSe^{\cdot}][H_2O]$ *(aq)* | 1165.3 | 1000546.4 | 699377.4 | 567029.696 | 0.9663 | -5.4200 | -4.8370 | 0.6994 | 0.6576 |
| $[RSe^{\cdot}][H_2O]$ *(g)* | 1201.3 | 95894.7 | 989122.5 | 362072.813 | 0.9658 | -5.6580 | -5.2450 | 0.9891 | 0.9279 |
| $RSe^{\cdot}$ *(aq)* | 1218.3 | 98258.3 | 911468.3 | 336981.625 | 0.9675 | -5.1941 | -4.7264 | 0.9115 | 0.8208 |
| $RSe^{\cdot}$ *(g)* | 1276.2 | 91848.0 | 1536358.9 | 543161.056 | 0.9712 | -5.1656 | -4.8863 | 1.5364 | 1.3797 |
| $CH_3Se^{\cdot}$ *(aq)* | 937.1 | 93299.9 | 1016002.7 | 370079.906 | 0.9686 | -5.1720 | -4.7440 | 1.0160 | 0.8979 |
| $CH_3Se^{\cdot}$ *(g)* | 994.5 | 86560.6 | 1850827.9 | 646127.672 | 0.9730 | -5.1840 | -4.9470 | 1.8508 | 1.6290 |
| $HSe^{\cdot}$ *(aq)* | 372.9 | 80689.6 | 951939.1 | 344333.878 | 1.0140 | -5.6640 | -5.1790 | 0.9519 | 0.8295 |
| $HSe^{\cdot}$ *(g)* | 506.0 | 75038.4 | 1781520.1 | 619021.509 | 1.0141 | -5.8990 | -5.6380 | 1.7815 | 1.5417 |

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# Appendix A

## Preparation of Rhenium-Modified Azurins

## Introduction

This appendix describes the methods of preparation of rhenium-modified mutant azurins that are common to the entire thesis. Mutant constructs for the H83Q/Q107H azurin were obtained as gifts from Cynthia Kiser.

## Materials and Methods

### Cell Growth.

All cells are grown in LB media. Depending on the vector, the cells will have resistance to the antibiotics ampicillin if the pET-3a vector is used or kanamycin if the pET-9a vector is used. The most commonly used cell strains are XL1-Blue, for cyclizing plasmid DNA and for DNA purification; BL21(DE3) and BL21*(DE3) for protein expression. No protein will be produced in the XL1-Blue cells.

Cells can be stored at –80 °C by the addition of sterile glycerol to a final concentration of 20 %. Thus, many of the cell strains (for example, for wild type azurin) were prepared by an earlier generation of graduate students. To revive these cells, use a sterile loop (available at the Biology Stockroom) to scrape the surface of the frozen cells (DO NOT THAW THE CELLS. THEY WILL DIE). Then, gently streak the loop with the cells on the surface of a LB plate with the appropriate antibiotic. The plates can be prepared in advanced and stored at 4 °C, however they must be equilibrated to room temperature before use. Place the plate in a 37 °C incubator; colonies should appear in

12-18 hours. After 24 hours, if no colonies are found, the plate should be discarded. At this point, the antibiotic will have been completely consumed or degraded. The information in the paragraph can be applied to any cell strain.

Using sterile forceps, pick up a sterile toothpick (from a dry autoclaved jar or toothpicks). Pick up one colony from the plate of cells. This is done by touching the toothpick on to the surface of a colony. Transfer the entire toothpick into a 10 mL Falcon tube that contains 4 mL of LB media (dilute 0.8 mL of the 5X LB by adding 3.2 mL sterile water) and 4 μL of 60 mg/mL ampicillin stock solution. The tube is then capped before being taken to a 37 °C shaker (shaking at 200 rpm). The cells are grown until the optical density (OD) at 600 nm of the cell containing media has reached 1.0. This usually takes at least seven hours for the BL21(DE3) or BL21*(DE3) strains, although the typical practice is to grow the 4 mL "starter" culture up to twelve hours, without adverse consequences to the cells. 4 mL cultures of XL1-Blue are usually grown for 16 hours to maximize the production of DNA.

Once the desired optical density has been reached, larger-scale preparations can be innoculated with cells from the starter culture. To 1 L of already autoclaved LB media, add the appropriate antibiotic (1 mL of the antibiotic stock solution gives a final concentration, in the case of ampicillin, of 60 μg/mL ampicillin). Then add 1 mL of the starter culture to the 1 L of LB media. This is known as a 1:1000 innoculation, which is standard for *E. coli*. Some other organisms do not grow as well and thus require a larger concentration of the starter culture for the innoculation. It is a good general practice whenever growing cells in a shaking incubator never to fill the flask more than 25 % of the total volume. Thus, it should be understood that 1 L of culture (as mentioned above)

has been prepared and autoclaved and will be used in a 4 L flask. Similarly, 1.5 L of culture are prepared and used in a 6 L flask. The same applies to smaller flasks. Thus, 250 mL of media will be used in a 1 L flask. The reason for this is so that the cells are properly aerated. With a 4 mL starter culture, there will be three 1 mL innoculations into three flasks containing 1 L of LB media (or two flasks containing 1.5 L of LB media). The flasks are then placed into a 37 °C incubator shaking at 200 rpm until the optical density of the cell containing media at 600 nm reaches 1.0. This takes about 7-8 hours. The optical density of 1.0 is the ideal, although in practice, high yields of protein are obtained when the optical density of the cells is between 1.0 and 2.0. The remainder of the starter culture is transferred to a 2 mL cryo vial (available at the Biology Stockroom). Autoclaved 100 % glycerol is then added to the cyro vial to give final concentration of 20 % glycerol. The mixture should be stirred gently with the pipette tip to ensure complete homogeneity of the 20 % glycerol containing cell stock. The vial is capped and then transferred immediately to the –80 °C freezer. This process is not necessary if there is already a glycerol containing frozen cell stock.

Once the larger scale cultures have achieved an $OD_{600}$ in the range of 1.0 to 2.0, protein expression is then induced by the addition of IPTG (isopropyl-β-D-thiogalactoside) stock solution (1 mL of the stock solution for 1 L of cell containing media). The cultures should remain in the shaking incubator for another four hours. During this time, the cells spend most of their energy making protein. The cells will continue to make protein beyond four hours, although azurin and many other overly expressed proteins can leak out of the cells into the media over a long period of time. It

is recommended that following the addition of IPTG that the cells not grow for more than five hours before harvesting.

Cells can be harvested by centrifuging the entire culture. 1 L centrifuge bottles that fit into a Beckman JLA-8.1 centrifuge are ideal for 3-6 L cultures because only one run is necessary. The balanced centrifuge bottles are centrifuged for 15 minutes at 6000 rpm at 4 °C. After centrifugation, the media is decanted into a large flask, leaving only the cell pellet behind. Before discarding the media, add bleach to the flask containing the decanted media. Swirl the mixture and let it sit for a few minutes. This is necessary to kill any residual bacteria in the media. Then, the bleached media can be poured into the sink.

The cell pellet is then resuspended in a one-tenth volume of a high osmolarity sucrose solution. One-tenth volume means this: if 3 L of cell culture were grown, 300 mL of the high osmolarity sucrose solution will be required. This is not a stringent requirement. 500 mL of this sucrose solution will work just as well. The suspension is then transferred into 300 mL centrifuge bottles (this is why 500 mL of the sucrose solution is also convenient, because the 500 mL of the resuspended solution can be divided evenly between two centrifuge bottles. The balanced bottles are then placed on a shaker for 15 minutes at room temperature. Afterwards, the centrifuge bottles are spun at 8000 rpm for 15 minutes. The supernatent is decanted; then, bleach is added to it before it is poured into the sink. The noticeably swelled cell pellet is then resuspended in a cold solution of 5mM $MgSO_4$ in water. This solution should be prepared in advanced and stored a 4 °C refrigerator or cold room until ready for use. The resuspended cells are shaken for 15 minutes at 4 °C and then are spun at 10000 rpm for 15 minutes. At this

stage, the supernatent should be saved. An osmotic shock on the cells has been performed, causing the cells to release the contents of the periplasm. Thus, the supernatent is known as the periplasmic extract. The cell pellet can be discarded. At this stage, the periplasmic extract contains: 5 mM $MgSO_4$ and the contents of the periplasm. To make the solution a buffer, 250 mM sodium acetate (buffered to pH 4.5 by acetic acid) is added to the periplasmic extrudate to give a final concentration of 25 mM sodium acetate. A white precipitant forms (attibutable to DNA and protein, including azurin aggregates). The merits of the acetate precipitation step are not well founded, although since the preparation yields enough azurin for subsequent experiments, no other alternatives have been explored. Following the acetate precipitation, the periplasmic extrudate is centrifuged at 8000 rpm for 15 minutes. The supernatent is saved; the white pellet is discarded. To the recovered supernatent is added either a solution 100 mM $CuSO_4$ or $ZnSO_4$ to give a final concentration of 10 mM $CuSO_4$ or $ZnSO_4$ (Ni or Co azurin can be prepared in the same way using the respective divalent sulfate salt solutions). The solution is left to sit overnight at 4 °C to allow for metal incorperation (from past experience, the day of cell growth and then osmotic shock ends late in the day, which is a convenient stopping point to go home for sleep). The solution containing Cu is blue; the solution containing Zn is clear (colorless). Since azurin can incorperate Zn, a metal present in the periplasm (while Cu is not present in the periplasm), the solution to which Cu has been added may contain a mixture of Cu and Zn azurin. It is advisable to determine the metal content of the azurin before any spectroscopic experiments are performed.

**Protein Purification.**

Following incorporation of the metal into azurin, the periplasmic extrudate is concentrated by Amicon filtration (using a YM10 cutoff membrane). Concentrated fractions are rinsed exhaustively with 25 mM sodium acetate, buffered to pH 4.5 until the effluent is colorless (excess metal has washed through) and until the absorption at 260 nm is close to zero (small bits of DNA having washed through). Azurin may be collected and stored in acetate buffer at 4 °C.

To purify azurin, the protein must be equilibrated with 20 mM diethanolamine (DEA), pH 9. This can be done by Amicon filtration by washing the protein that is originally in acetate buffer with the DEA buffer until the effluent reaches pH 9. At this point, the amount of acetate in the protein solution is negligible. Azurin will then be loaded on to a MONO Q column (Q stands for quaternary amine—this column is an anion exchanger). The MONO Q column is hooked up to the FPLC setup (see Appendix B for instructions on using the FPLC). For MONO Q, buffer A is 20 mM DEA, pH 9 and buffer B is 20 mM DEA, 200 mM NaCl, pH 9. The column is washed with three column volumes of buffer B followed by three column volumes of buffer A. After loading the protein in the superloop (valve position 1.1), it is loaded onto the column (valve position 1.2) with buffer A. For a 10/10 column, about 10 mg can be loaded on to the column for purification. Once the protein is loaded on to the column, the programmed gradient can be started. From 0 % B, the gradient is increased to 10 % B in 2 mL. The gradient is then held at 10 % B until the intensity of the eluted bands (at 255 nm) returns close to the baseline (this usually takes about 6 mL). Azurin is still bound to the column at this point,

so anything eluted can be discarded. The gradient is then increased to 15 % in 2 mL and held. Azurin elutes at 15 % B (30 mM NaCl), so the one band that appears at this concentration of buffer B is the azurin of interest. After azurin has been eluted, the gradient is continued to 100 % buffer B. Other peaks appear between 15 and 100 % B. These are not azurins. They may be discarded. The eluted azurin is collected, concentrated, equilibrated with HEPES buffer, and stored until ready for use.

**Preparation of Rhenium Complexes.**

$Re(CO)_5Cl$ and the diimine are dissolved in toluene in a 1:2 molar ratio. This mixture is then heated to 60 °C for 5 hours. The solution will change color from white to dark yellow. The solution is then concentated by rotary evapoation. The yellow solid is then redissolved in $CH_2Cl_2$ ($Re(CO)_5Cl$ is insoluble in $CH_2Cl_2$) and filtered. The $Re(CO)_3$(diimine)Cl is then precipitated by addition of n-pentane.

The first step of the prep is described in a paper by Mark Wrighton (J.Am.Chem.Soc. 1974, volume 96, pages 998-1003) who modified the original synthesis of Geoffrey Wilkinson (J.Chem.Soc. 1959, pages 1501-1505). A slight excess of the diimine is mixed with Re(CO)5Cl in benzene or toluene and heated at 60 °C for a couple of hours. The starting rhenium compound is white; phenanthroline is also white, while the 4,7-dimethylphenanthroline is dull and golden. I usually did this on the scale of 0.5 g Re(CO)5Cl (1.4 mmol) and 0.4 g diimine (~2 mmol) in 30 mL of toluene. You can increase or decrease the scale of the reaction without any problem, if you want; you do not need to carry this reaction out under an inert atmosphere, but I would make sure that

you have a condenser above your flask so that you do not accidentally boil off your solvent. After heating (while stirring) this mixture, it will become bright yellow. The reaction is complete after three or four hours. The reason it takes this long is because Re(CO)5Cl is only slightly soluble in toluene or benzene, so it takes a while for the rhenium to react with the readily soluble diimine. I have set up the reaction before going home in the evening and then worked up the product the next morning, so it is not a problem to let the reaction go longer than just a couple of hours. The tricarbonylrhenium diimmine is practically insoluble in benzene or toluene, so you will see a yellow precipitant at the bottom of your flask. You can filter this solid off immediately and rinse the solid with n-pentane (to dissolve off any remaining free diimine). Again, the tricarbonyl rhenium diimine is sparing soluble in pentane and any hydrocarbon solvent, so you don't have to worry about losing your product in the rinse. The yield on this reaction is high >80%. Mark Wrighton's paper (mentioned earlier) and a follow up paper (J.Am.Chem.Soc. 1978, volume 100, pages 5790-5795) describe the photophysical and excited state properties of the tricarbonyl rhenium diimine halides.

The next step in the synthesis requires the removal of the halide. You can either do that by abstracting the halide with a silver salt (silver triflate, for example) or by protonating the chloride with a strong acid. I have experience with the latter, which comes from a prep by Sullivan and Meyer (J.Chem.Soc. Chem.Commun. 1984, pages 1244-1245). The yellow solid, Re(CO)3(diimine)Cl (0.5 g) is added to 30 mL of dichloromethane. When you stir this mixture, you will see it is a slurry. In other words, the rhenium compound is mainly suspended in the solvent, but not very well dissolved. Then, you will add dropwise pure triflic acid (trifluorosulfonic acid). I would buy this

from Fluka, because the bottle has a screw cap. If you buy triflic acid from Aldrich, it comes in ampules, which you would have to score and break to get to the acid. Of course, it is more difficult to close a open ampule to store the triflic acid for later. Triflic acid is extremely hygroscopic, so you will notice that when you open the bottle of the acid, it will begin to fume. Also, be EXTREMELY careful when handling this acid. Triflic acid has a pKa of -13, so it can protonate chloride to make hydrochloric acid. This is the point of using it in this reaction. Triflic acid will protonate the coordinated chloride, which will then dissociate from the rhenium. Hydrochloric acid will bubble away and the triflate anion will take its place. Well, that's enough about the explanation. I should get back to the details of the experiment. Once you have made the slurry of $Re(CO)_3$(diimine)Cl in dichloromethane, you will add triflic acid dropwise using a pasteur pipet until all of the solution becomes transparent (the rhenium is now in solution). You will add about 1 mL total. Just remember to add the triflic acid dropwise, and not in one shot. Now, you will leave the reaction to stir at room temperature for about 3 or 4 hours. You will know when the reaction is finished when all of the HCl gas has bubbled away. You can determine this by suspending a wet (by water) piece of pH paper just over the surface of the reaction. Once the pH approaches 7, the reaction is done. Then, add diethylether dropwise (you can add the ether faster than the acid, but I would not add 30 mL of ether in one shot). At this point, a yellow solid will precipitate out. This will be mainly the $Re(CO)_3$(diimine)(triflate) with some of the starting material, $Re(CO)_3$(diimine)Cl as an impurity (both are yellow). Collect this yellow solid and rinse it with ether. If you add the $Re(CO)_3$(diimine)(triflate) to water, water itself will undergo a ligand substitution reaction do displace the triflate to the outer sphere. Thus, add the

yellow solid from the previous reaction into a beaker of water; then, heat the beaker. The triflate adduct will take a while to dissolve into the warm water (~60-70 C); the chloride impurity left over from the previous step is not soluble in water, and that will just float at the top of the beaker. You should filter off the undissolved yellow solid (let the solution stir for about an hour) and you will be left with a tranparent and orange solution. This is the $Re(CO)_3(diimine)(H_2O)^+$ species. When the diimine is phenanthroline, the compound is saturated at 5 mM in water (at room temperature); the 4,7-dimethylphenanthroline analog saturates at 2 mM. So what you want to do is to slowly boil of most of water and then cool the mixture down to room temperature. The $Re(CO)_3(diimine)(H_2O)$][triflate] will precipitate as orange crystals. Now, you can collect the crystals and store them until you are ready to do the rhenium labeling reactions on the protein.

**Rhenium-Labeling Reactions.**

The procedure is published in the supporting information of the Di Bilio et al. paper (JACS, volume 123, 2001, pages 3181-3182).

Before labeling, the protein is equilibrated in 25 mM HEPES buffer, pH 7.2 (you don't want to have a solution much more basic than this, because you would deprotonate the coordinated water on the rhenium, giving you now the rhenium hydroxy species which is insoluble in water). You should concentrate the protein as much as possible (the most concentrated that I have been able to achieve is 6 mM = ~85 mg/ml, but for the labeling, I use anywhere between 2-6 mM). You would then dilute your protein sample

in HEPES buffer 15 fold with your rhenium complex dissolved in water. Since the (4,7-dimethylphenanthroline)- (tricarbonyl)rhenium aquo species saturates at 2 mM, you would have up to 0.9 mM azurin in with 1.8 mM rhenium in your reaction mixture. You can set up the reaction in the 15 or 50 mL conical vials (I'm sorry to go into so much detail on this; the bottom line is that you can set you can set up the reaction in any container and on any scale). I let the reaction sit for a week at 37 C. Some rhenium will inevitably preciptate out, however, you added excess rhenium, so the labeling should still go. I should point out that when you make your stock rhenium solution you will have to heat the sample and stir gently until the solid dissolves. You will not be able to dissolve the rhenium compound at room temperature in a reasonable amount of time. After the one week, I concentrate the sample so that the unreacted rhenium washes through. I then run a gel filtration column (containing Sephadex G-10) equilibrated in 25 mM sodium acetate (buffered to pH 4.5 with acetic acid) to separate out the remaining rhenium. At this point, I let the protein sit overnight at 4 C, at the very least, although longer cannot hurt. Acetate can pull off some rhenium mislabeled at glutamates or aspartates. Now you can equilibrate the protien in 20 mM NaPi, 750 mM NaCl, pH 7.2 in preparation for the IMAC column. Here again, I would let this sit overnight at 4 °C in case there is still rhenium that is going to precipitate.

# Appendix B

## Photochemical and Spectroscopic Methods

## INTRODUCTION

## MATERIALS AND METHODS

### Freeze-Flash Quench Generation and Trapping of Radicals.

All photochemical methods and electron transfer experiments are described in detail in the thesis of Jeremiah Miller.[1]

### X-Band EPR.

The X-band EPR setup at Caltech is described in the paper by Di Bilio *et al.* [2]

**High-Field (HF) EPR.** EPR spectra at high magnetic fields were recorded at the Commissariat à l'Energie Atomique (CEA) Saclay in France in collaboration with Professor Sun Un. The magnetic field was swept by a 10.5 T magnet at two static microwave frequencies, 190 and 285 GHz. The experimental setup and the simulations of the spectra have been described in the literature.[3]

**References**

(1) Miller, J. E. *Ph.D. Thesis*; California Institute of Technology: Pasadena, 2003.
(2) Di Bilio, A. J.; Crane, B. R.; Wehbi, W. A.; Kiser, C. N.; Abu-Omar, M. M.; Carlos, R. M.; Richards, J. H.; Winkler, J. R.; Gray, H. B. *J. Am. Chem. Soc.* **2001**, *123*, 3181-3182.
(3) Ivancich, A.; Mattioli, T. A.; Un, S. *J. Am. Chem. Soc.* **1999**, *121*, 5743-5753.

# Epilogue

All scientific ideas are built upon the work of others. Moses Gomberg, who discovered the triphenylmethyl radical, served as the PhD advisor for John Bailar. Bailar, who is recognized as the father of coordination chemistry in the United States, studied the stereochemistry of inorganic complexes and served as the PhD advisor to Fred Basolo. Basolo became interested in the mechanisms of ligand substitution in transition metal complexes. His book on *Mechanisms of Inorganic Reactions* (2nd Edition, 1971) co-authored with Ralph Pearson is a classic work in the field.

In the late 1950s, Basolo and Pearson served as the PhD advisors to Harry Gray, whose thesis was on the mechanism of ligand substitution of square-planar platinum complexes. Since then, for more than forty years, Harry has advanced the frontiers of chemistry, particularly in the study of the electronic structures of inorganic and bioinorganic complexes and in the kinetics and mechanism of inorganic and bioinorganic processes. In addition, along with Jay Winkler, Harry applied his interests in inorganic photochemistry to the problems of long-range electron transfer in proteins and to the study of the dynamics of protein folding. This is a small sampling of Harry's amazing work.

The Century of chemistry by these four chemists—Gomberg, Bailar, Basolo, and Gray—paved the way for the next generation of chemists, which includes me. It is humbling to be a part of this lineage and to see where my contribution to science fits within the Bigger Picture.