

**ELECTRON TUNNELING AND HOPPING THROUGH PROTEINS**

**Thesis by**  
**Crystal Shih**

In Partial Fulfillment of the Requirements for  
the Degree of Doctor of Philosophy

California Institute of Technology

Pasadena, California

2008

(Defended May 5, 2008)

© 2008

Crystal Shih

All Rights Reserved

## ACKNOWLEDGMENTS

I've loved science ever since I was in the third grade, but as I also "loved" New Kids on the Block, my opinion was not really to be trusted. However unwise I was in some choices, I had fantastic teachers, among them Karen Wickersham, Heston Bates, and Ross Graham, to take my interest and root it in science.

My interest in chemistry intensified when I arrived at college and had the fortune of having the irrepressible Phil Miller as my TA for organic chemistry. Phil inspired me to change my major from biology to chemistry, and I will always be grateful to him for his advice.

I joined Greg Fu's lab in spring of 2002. Greg is not only a brilliant teacher, but also a considerate advisor; I've really appreciated his guidance and support. My mentor in the group, Brian Hodous, provided a good deal of laboratory common sense and technique, as well as hours of smiles and entertainment. I am indebted to Ryo Shintani; not only did he help me with my experiments and Japanese homework countless times, but he's also continued to provide me with friendship, inspiration, and very solid advice whenever I've needed it.

However brief my experience was in Dave MacMillan's lab, I want to thank Roxanne Kunz, Alan Northrup, and Joel Austin for making it fun and educational. I don't think I'll ever come across a baymate as fun, clever, or driven as Ian Mangion.

And now, I'm at the meat of the order: Harry Gray, Jay Winkler, and my friends in the Gray Group. Harry's positive attitude, insights, and generosity have carried me through confusing results and dry spells. He's changed my perception of academics, fed

my Dodger addiction, and taught me the importance of keeping steady, holding my head high, and "not peaking too soon". Jay Winkler dragged me into the rocky world of MATLAB, and I am very thankful for his guidance. I've appreciated his patience and support while I was learning the ropes, as well as our enlightening discussions.

I've interacted with a number of interesting and fun people in the Gray group, and I appreciate their being here to make life different and exciting every day. Brian Leigh's hours of instruction have been invaluable over the course of my grad school career; his generosity, patience, and sense of humor have been indispensable to my sanity at especially rough patches. Yen Hoang Le Nguyen's zeal for life kept me inspired, and her discussions about all things, from knitting to my currently non-working reaction, always kept me on my intellectual toes. Jillian Dempsey's been a fantastic roommate, officemate, co-TA, and friend, always ready with a smile and good advice. Gretchen Keller's positive attitude makes her such a great person to be around; planning parties with her has been fun. A conversation with the cheerful, sweet, and enthusiastic Heather Williamson unfailingly gives me an extra shot of motivation when my energy starts to flag. I love Nicole Bouley's energetic enthusiasm for life (and Jane Austen)!! Keiko Yokoyama's smiles and steady company unfailingly cheer me up when I'm frustrated, and I adore her taste in fine art. Lionel Cheruzel's witty mind, incredible work ethic, and modesty are to be marveled at. Too bad he's French. ;) I'd especially like to thank him for reading through a portion of my thesis. Kyle Lancaster is a talented mixer; "Equilibrium" got me through props, and I enjoy his interesting taste in music. Alec Durrell is fun and funny company during exhausting and annoying temperature studies. When I joined the lab, Mayra Sheikh, a very talented undergrad, had more experience in

the Gray group than I, and I really enjoyed learning from and joking with her. My own SURF of two years, Deepak Mishra, has been fun to collaborate with, (being the triple threat of motivated, intelligent, *and* independent!) despite his inherent flaw of being a chemical engineering major.

I'd argue that no other class in the Gray Group takes as much pride and love in each other as mine. As my first party co-coordinator, as well as one of the Nanosecond-I GLAs, Don Walker has had the burden of dealing with me at my most anal and most stressed out, and he's unfailingly calmed me down with his relaxed and steady attitude. While she's extremely intelligent, I still think Melanie Pribisko Yen's got to be more than 75% heart, because I haven't seen anyone so strong, courageous, and generous. I'm especially grateful for her friendship this last year, what with our picosecond trial by fire and long march to graduation. As well as being my permanent officemate, Bert Lai is the second little brother I never had. He's been a constant source of comfort when things go south, and he's one of the best wingmen to have when flying high.

I would not have been able to do half the experiments or studies I'd wanted to do without Yuling Sheng's assistance, advice, and friendship. She's made working with protein *fun*, a development I'd never expected! I am especially thankful to her for taking on the burden of carrying out mutations and expressing the bulk of the proteins.

I am also extremely thankful to Angel Di Bilio, for his advice on labeling and purification protocols, as well as his unique and helpful perspective.

Outside of lab, I've met a number of really great people at Caltech. Steven Baldwin's a great guy to go to Dodger games with, and one of only two people I'd ever trust with My Scorebook. Baseball aside, he's a great friend, and I'm thankful to have

him here to support me with an insightful scientific discussion, a margarita, or a much-needed trip to Borders.

Through first year classes, candidacy, bad lab days, laser time, and tons of travel, Anna Folinsky, Jennifer Roizen, and I have kept to our Thursday Lunch routine, at times also entertaining illustrious guests such as the Pasadena PD (uninvited) and Brian Stoltz (very welcome). I not only learned a lot about Jewish culture, but I also discovered a lot about myself in our varied and interesting discussions. I am especially grateful to Anna for her unique personality, interesting opinions, and fierce support. Anna tells it like it IS!!! I'd also like to thank our occasional guests, Jennifer Stockdill and Erin Koos for their fun opinions and warm hearts.

Finally, a number of people outside of my Caltech life have ensured that beyond having just *having* a life, I'm having an absolutely *fantastic* one. First, the Los Angeles Dodgers are great to watch when you've just messed up a whole crop of labeled protein and have no idea what to do with your latest laser data. The Jane Austen Society of North America has a fantastic contingent here in Southern California, and I've especially loved participating in the Pasadena reading group for the fun non-scientific discussion. The PCG have nurtured my love for writing, and I'm glad to have their encouragement in all things, from my writer's block to my next laser study. They kept me fantastic company while I was marching away on the thesis, and I am grateful to them for their support, and for remembering flippy. I'd also like to thank my many friends at DWG. Their encouragement and perspective has been invaluable. Having Jennifer Santucci and Victoria Young only a drive away, and always up for an adventure, has done much to give me peace of mind when I most needed it. Alyson Lee has been a constant

inspiration to me and I've appreciated our many discussions, as well as her unflagging support. Amy Ishizawar should get thousands of pounds of mochi, because she's been absolutely indispensable (not findispensable!) in keeping me laughing and sane throughout grad school, and especially through this last push towards graduation.

Margaret Douglass, ever in Europe, but ever on-line and supportive, is always ready to pitch an article on elephants to me when I need a break (and even when I don't need one). Our trip through Austria will always be one of the best trips I've ever had in my life. The Next Fifth East crew from college has been extremely generous in accommodating my circumstances and schedule when planning get-togethers. I am especially grateful to Flora Lee, Roland Burton, and Corinna Sherman for taking time out of their busy schedules to check in and/or visit.

"My boyfriend, the doctor" Jeffery Byers had me at the words "ligand field splitting". An honest opinion when I didn't want but needed it, a fountain of chemical and baseball knowledge, a partner-in-crime to escape campus with, he is the best friend I've ever had, and I am extremely grateful to him for his support over the years.

Finally, I'd like to thank my family for their love and generosity. My cat Lina always knows when I need a good cuddle. My brother Terry has always inspired me to push harder and question everything, and his unflappable strength is very easy to lean on when things get difficult. I am especially grateful to him for his visits out to see me, as he is quite busy himself. My parents have done more for me and my brother than I think *can* be done, and I hope they know how grateful I am to have such intelligent, interesting, loving, and compassionate people as my care-takers and role models. It is to them I dedicate this thesis.

*This thesis is dedicated to my Mom and Dad,  
for being the best inspiration a person could have.*

## THESIS ABSTRACT

Long-range electron tunneling is a central component of processes that are essential for biological function. While many studies have been made to understand electron transfer in proteins, biologically efficient electron transfer at distances exceeding 25 Å remains unobserved in these experiments and hence unresolved. It is proposed that long-range electron transfer is in actuality multistep electron tunneling. What is reported in this thesis is the design, synthesis, and study of many protein systems for the purpose of studying multistep electron tunneling in azurin.

In each system, a histidine has been introduced on the protein for attachment of a high-potential ruthenium or rhenium sensitizer ( $[\text{Ru}(\text{trpy})(\text{tfmbpy})]^{2+}$  or  $[\text{Re}(\text{dmp})(\text{CO})_3]^+$ ); a nitrotyrosine, tryptophan, or tyrosine is placed between the two metal centers on the tunneling pathway. The electron transfer is triggered with the excitation of the metal label with laser light, and the kinetics are monitored, for the most part, by time-resolved UV-VIS spectroscopy.

The first system to empirically demonstrate multistep electron tunneling in proteins was discovered; ultrafast electron transfer is observed between the copper and rhenium centers in the Re124/W122 system; the system was structurally characterized and studied by time-resolved UV-VIS and IR spectroscopies. A two-step tunneling model is proposed; the data sets for the different methods utilized are all in excellent agreement with the model.

Systematic perturbations were made to the working hopping system. It was discovered that nitrotyrosine can participate as an intermediate, but studies to

demonstrate its participation in multistep tunneling are not yet fully realized. A second hopping system was discovered in the development of the Re126/W122 system.

## TABLE OF CONTENTS

Acknowledgment .....	iii
Dedication .....	viii
Thesis Abstract.....	ix
Table of Contents .....	xi
List of Tables, Figures, and Schemes .....	xiv
<b>Chapter One: Background &amp; Research Plan .....</b>	<b>1</b>
1.1 Statement of Intent.....	1
1.2 Electron Transfer in Proteins .....	1
Electron Transfer Theory .....	1
Electron Transfer Experiments: Metal-Modified Metalloproteins.....	5
1.3 Long-Range Electron Transfer in Proteins.....	9
1.4 Protein-Based Radicals .....	11
1.5 Multistep Tunneling in the Gray Group.....	13
1.6 Research Outline.....	15
High-Potential Ruthenium Sensitizers .....	15
3-Nitrotyrosine as an Intermediate.....	15
Hopping Systems .....	16
1.7 References .....	18
<b>Chapter Two: Preparation &amp; Characterization of Ru<sup>2+</sup>- and Re<sup>+</sup>-modified</b>	
<b><i>Pseudomonas aeruginosa</i> Azurins .....</b>	<b>21</b>
2.1 Abstract .....	21
2.2 Introduction .....	21
Design of Hopping Systems.....	21
<i>Pseudomonas aeruginosa</i> Azurin.....	22
Reduction Potentials & Photosensitizers.....	24
Chapter Outline.....	27
2.3 Results & Discussion .....	28
Metal Labels .....	28
[Ru(tfmbpy) <sub>2</sub> (im) <sub>2</sub> ] <sup>2+</sup> .....	28
The 3-2-1 Architecture.....	30
Rhenium .....	36
Azurin .....	36
Site-Directed Mutagenesis & Expression of Mutants.....	36
Nitration of Tyrosine.....	37
Unfolding Studies .....	39
Metal-Labeled Azurin.....	46
[Ru(tfmbpy) <sub>2</sub> (im)(HisX)] <sup>2+</sup> .....	46
[Ru(trpy)(tfmbpy)(HisX)] <sup>2+</sup> .....	47
[Re(dmp)(CO) <sub>3</sub> (HisX)] <sup>+</sup> .....	48

	Labeled Proteins.....	49
2.4	Conclusions .....	49
2.5	Experimentals .....	50
	Materials .....	50
	Resources & Instrumentation .....	51
	Synthesis & Characterization of Ruthenium Model Compounds .....	51
	4,4'-bis(trifluoromethyl)-2,2'-bipyridine (tfmbpy) .....	51
	Ru(tfmbpy) <sub>2</sub> Cl <sub>2</sub> ·2H <sub>2</sub> O .....	52
	[Ru(tfmbpy) <sub>2</sub> (im) <sub>2</sub> ](PF <sub>6</sub> ) <sub>2</sub> .....	53
	[Ru(trpy)(tfmbpy)Cl]Cl.....	54
	[Ru(trpy)(tfmbpy)(im)](PF <sub>6</sub> ) <sub>2</sub> .....	54
	[Ru(trpy)(bpy)Cl]Cl .....	55
	[Ru(trpy)(bpy)(im)](PF <sub>6</sub> ) <sub>2</sub> .....	55
	Ru(Cl-trpy)Cl <sub>3</sub> .....	56
	[Ru(Cl-trpy)(bpy)Cl]Cl.....	56
	[Ru(Cl-trpy)(bpy)(im)](PF <sub>6</sub> ) <sub>2</sub> .....	57
	Protein Protocols.....	57
	Site-Directed Mutagenesis .....	57
	Expression of Mutant Proteins .....	57
	Nitration of Tyrosine.....	58
	Labeling Protein.....	59
	Purification of Azurin .....	61
	Chelating Column .....	61
	Cation-Exchange Chromatography .....	63
	Anion-Exchange Chromatography .....	65
	Electrochemical Measurements .....	67
	Circular Dichroism Measurements.....	67
	Laser Spectroscopy & Analysis .....	68
	Sample Preparation .....	68
	Data Analysis.....	69
2.6	References .....	70
<b>Chapter Three: Dramatic Acceleration of Electron Flow through Azurin.....</b>		<b>72</b>
3.1	Abstract .....	72
3.2	Introduction .....	72
	The System .....	72
	Chapter Outline.....	73
3.3	Results & Discussion .....	74
	Time-Resolved UV-VIS Spectroscopy with a 10 ns Laser.....	74
	Structural Characterization .....	75
	Time-Resolved IR Spectroscopy.....	76
	Temperature Studies .....	78
	Time-Resolved UV-VIS Spectroscopy with a 10 ps Laser.....	79
	The Model .....	80
	Hopping Map.....	81
3.4	Conclusions .....	83

3.5	Experimentals .....	83
	Collaborators .....	84
	Temperature Studies .....	84
	Laser Spectroscopy with a 10 ps Laser .....	84
	Data Analysis .....	84
3.6	References .....	87
<b>Chapter Four: Controlling Electron Hopping .....</b>		<b>88</b>
4.1	Abstract .....	88
4.2	Introduction .....	88
	The System .....	88
	Chapter Outline .....	89
4.3	Results & Discussion .....	89
	The Importance of Reduction Potentials in Hopping .....	89
	Ru124/W122/Az(Cu <sup>2+</sup> ) .....	90
	Re124/YNO <sub>2</sub> 122/Az(Cu <sup>2+</sup> ) .....	92
	Ru124/YNO <sub>2</sub> 122/Az(Cu <sup>2+</sup> ) .....	94
	The Importance of Distance in Hopping .....	97
	Ru126/W122/Az(Cu <sup>2+</sup> ) .....	97
	Re126/W122/Az(Cu <sup>2+</sup> ) .....	98
4.4	Conclusions .....	100
4.5	Experimentals .....	100
4.6	References .....	101
<b>Chapter Five: Studying Hopping in Another Pathway in Azurin .....</b>		<b>102</b>
5.1	Abstract .....	102
5.2	Introduction .....	102
5.3	Results & Discussion .....	103
5.4	Conclusions .....	105
5.5	Experimentals .....	106
5.6	References .....	106
<b>Chapter Six: Future Directions .....</b>		<b>107</b>
6.1	Systems to Study .....	107
6.2	Thesis Conclusions .....	108
<b>Appendix: MATLAB Programs and Functions Utilized in Data Analysis.....</b>		<b>110</b>

## LIST OF TABLES, FIGURES, AND SCHEMES

### Chapter One: Background & Research Plan

Figure 1.1	Potential energy curves .....	2
Figure 1.2	Electron transfer between $\text{Ru}(\text{NH}_3)_5^{2+}$ and $\text{Fe}^{3+}$ .....	6
Figure 1.3	Driving force dependence of electron transfer rates in Ru-His33 Cytochrome <i>c</i> .....	7
Figure 1.4	Tunneling timetable .....	8
Figure 1.5	Distance dependences of observed electron transfer rates in cytochrome <i>c</i> oxidase and bacterial photosynthetic reaction centers .....	10
Figure 1.6	Possible plan for studying multistep tunneling in proteins .....	13
Figure 1.7	Hopping residues investigated .....	14
Table 1.1	Measured reduction potentials for amino acids .....	12

### Chapter Two: Preparation & Characterization of $\text{Ru}^{2+}$ - and $\text{Re}^+$ -modified *Pseudomonas aeruginosa* Azurins

Figure 2.1	Two possible hopping systems .....	22
Figure 2.2	Crystal structure of <i>Pseudomonas aeruginosa</i> azurin .....	23
Figure 2.3	Reduction potentials and $\text{pK}_a$ s of relevant oxidation/protonation states of tryptophan and tyrosine .....	26
Figure 2.4	Modified Latimer diagram of $\text{Re}(\text{phen})(\text{CO})_3^+$ .....	27
Figure 2.5	Absorption and emission spectra of $[\text{Ru}(\text{tfmbpy})_2(\text{im})_2](\text{PF}_6)_2$ in acetone .....	30
Figure 2.6	Time-resolved emission of $[\text{Ru}(\text{tfmbpy})_2(\text{im})_2](\text{PF}_6)_2$ in water .....	30
Figure 2.7	UV-VIS spectra of three ruthenium sensitizers in acetonitrile .....	32
Figure 2.8	Absorption and emission spectra of $[\text{Ru}(\text{trpy})(\text{tfmbpy})(\text{im})](\text{PF}_6)_2$ in acetonitrile .....	33
Figure 2.9	Cyclic voltammograms of three ruthenium sensitizers .....	34
Figure 2.10	Modified Latimer diagram of $\text{Ru}(\text{trpy})(\text{tfmbpy})(\text{im})$ .....	35

Figure 2.11	Emission data: Ru(trpy)(tfmbpy)(im) <sup>2+</sup> .....	35
Figure 2.12	Sequence of All-Phe azurin.....	37
Figure 2.13	UV-VIS spectrum of successful nitration.....	38
Figure 2.14	UV-VIS spectra of azurin in increasing concentrations of methanol .....	41
Figure 2.15	CD spectra of azurin in increasing concentrations of methanol....	41
Figure 2.16	UV-VIS spectra of azurin in 50% methanol over time.....	42
Figure 2.17	UV-VIS spectra of azurin in increasing concentrations of urea....	43
Figure 2.18	Zoom in of 500–800 nm region of Figure 2.17 .....	43
Figure 2.19	CD spectra of azurin in increasing concentrations of urea .....	44
Figure 2.20	UV-VIS spectra of azurin in increasing concentrations of urea, 24 hours .....	45
Figure 2.21	Zoom in of 500–800 nm region of Figure 2.20 .....	45
Figure 2.22	CD spectra of azurin in increasing concentrations of urea, 24 hours .....	46
Figure 2.23	UV-VIS spectrum of [Ru(trpy)(tfmbpy)] <sup>2+</sup> -labeled azurin.....	48
Table 2.1	Estimated reduction potentials for redox couples relevant to multistep electron tunneling studies .....	25
Table 2.2	Overall yields and overall time taken to synthesize three 3-2-1 architecture ruthenium compounds .....	32
Table 2.3	Reduction potentials of three ruthenium sensitizers .....	34
Table 2.4	Labeled proteins studied in this dissertation.....	49
Table 2.5	Solutions for HiTrap chelating column .....	61
Table 2.6	Method utilized to purify labeled proteins on the chelating column .....	62
Table 2.7	Methanol gradient method run to remove metal labels from chelating and ion-exchange columns.....	63
Table 2.8	Solutions for Mono S column .....	63

Table 2.9	Method utilized to purify azurin on the Mono S column.....	64
Table 2.10	Solutions for Mono Q column.....	65
Table 2.11	Method utilized to purify azurin on the Mono Q column.....	66
Scheme 2.1	Synthesis of $[\text{Ru}(\text{tfmbpy})_2(\text{im})_2](\text{PF}_6)_2$ .....	28
Scheme 2.2	Synthesis of 3-2-1 ruthenium compounds.....	31
Scheme 2.3	Nitration of tyrosine.....	38
Scheme 2.4	Generation of $\text{Ru}(\text{tfmbpy})_2\text{CO}_3$ and installation onto azurin.....	46
Scheme 2.5	Generation of $[\text{Ru}(\text{trpy})(\text{tfmbpy})(\text{H}_2\text{O})]\text{NO}_3$ followed by installation onto azurin.....	47

### Chapter Three: Dramatic Acceleration of Electron Flow through Proteins

Figure 3.1	<i>Pseudomonas aeruginosa</i> azurin.....	73
Figure 3.2	Transient absorption of $\text{Re124/W122/Az}(\text{Cu}^+)$ .....	75
Figure 3.3	Crystal structures of Re-labeled azurins.....	76
Figure 3.4	Difference time-resolved IR spectra of $\text{Re124/W122/Az}(\text{Cu}^+)$ .....	77
Figure 3.5	Fluorescence spectra for Re and $\text{Re/W122/Az}(\text{Cu}^+)$ at 25°C and -20°C.....	78
Figure 3.6	Time-resolved emission of $\text{Re124/W122/Az}(\text{Cu}^+)$ .....	79
Figure 3.7	Two-step hopping map for electron tunneling through Re-modified azurin.....	82
Figure 3.8	Instrument response data fit to Gaussian functions.....	85
Figure 3.9	A figure generated using 'fminsearch' and function 'crystal'.....	87
Scheme 3.1	Events after sample excitation.....	74
Scheme 3.2	Kinetics model of photoinduced electron transfer in $\text{Re124/W122/Az}(\text{Cu}^+)$ .....	80

### Chapter Four: Controlling Electron Hopping

Figure 4.1	Time-resolved emission of $\text{Ru124/W122/Az}(\text{Cu}^{2+})$ and $(\text{Cu}^+)$ .....	90
Figure 4.2	Transient absorption of $\text{Re124/W122/Az}(\text{Cu}^{2+})$ and $(\text{Cu}^+)$ .....	91

Figure 4.3	Time-resolved emission of Re124/YNO <sub>2</sub> 122/Az(Cu <sup>+</sup> ).....	92
Figure 4.4	Time-resolved UV-VIS spectroscopy of Ru124/YNO <sub>2</sub> 122/Az(Cu <sup>2+</sup> ) at pH 4.7 and 7.7 .....	95
Figure 4.5	Time-resolved UV-VIS spectroscopy of Ru124/YNO <sub>2</sub> 122/Az(Cu <sup>+</sup> ) at pH 4.7 and 7.7 .....	96
Figure 4.6	Time-resolved emission of Ru126/W122/Az(Cu <sup>2+</sup> ) and (Cu <sup>+</sup> ).....	97
Figure 4.7	Time-resolved emission of Re126/W122/Az(Cu <sup>2+</sup> ) and (Cu <sup>+</sup> ).....	98
Figure 4.8	Transient absorption of Re126/W122/Az(Cu <sup>+</sup> ) with quencher.....	99
Scheme 4.1	Events after sample excitation in Ru124/W122/Az(Cu <sup>2+/+</sup> ).....	92
Scheme 4.2	Events after sample excitation in Re124/YNO <sub>2</sub> 122/Az(Cu <sup>+</sup> ) .....	94
Scheme 4.3	Events after sample excitation in Re126/W122/Az(Cu <sup>+</sup> ) with Ru(NH <sub>3</sub> ) <sub>6</sub> <sup>3+</sup> .....	100

### Chapter Five: Studying Hopping in Another Pathway of Azurin

Figure 5.1	<i>Pseudomonas aeruginosa</i> azurin.....	103
Figure 5.2	Time-resolved UV-VIS spectroscopy of Ru83/Y83/Az(Cu <sup>2+</sup> ) ...	104
Figure 5.3	Time-resolved emission of Ru83/Y48/Az(Cu <sup>+</sup> ) .....	104
Scheme 5.1	Events after sample excitation in Ru83/Y48/Az(Cu <sup>2+</sup> ).....	105

### Chapter Six: Future Directions

Table 6.1	Azurin mutants which were expressed but not discussed in this dissertation.....	107
-----------	---------------------------------------------------------------------------------	-----