

**SELECTIVE FUNCTIONALIZATION OF THE
PROTEIN N-TERMINUS WITH
N-MYRISTOYL TRANSFERASE IN BACTERIA**

Thesis by
Chethana Kulkarni

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



CALIFORNIA INSTITUTE OF TECHNOLOGY
Pasadena, California

2013
(Defended October 5, 2012)

© 2013

Chethana Kulkarni

All Rights Reserved

ACKNOWLEDGEMENTS

Every Ph.D. thesis is the product of the efforts of many individuals, and my thesis is no exception. Upon reaching this point in my education and my career, I feel very fortunate to have benefited from the knowledge, inspiration, support, guidance, and good will of many people at different stages of my life. I found that the most straightforward manner in which to organize my thoughts, and this list, was chronologically.

First and foremost, I am greatly indebted to my family for their incredible sacrifices and unwavering support in all aspects of my education. My parents left behind everything and everyone they knew in India to make a better life for themselves and their daughters here in the United States. I could never imagine undertaking such a move myself, and I can never thank them enough for the endless love and generosity they have shown to my sister and me. For my entire life, my sister has been my most vigorous cheerleader. Her fiery spirit, sense of humor, strong will, and big heart inspire me.

I am grateful for the many wonderful teachers and mentors I had before coming to Caltech. In high school, my AP Chemistry teacher, Mr. James Flores, applied an unparalleled love of chemistry, commitment to teaching, and general rigor to every aspect of our coursework. I would not have majored in chemistry in college had it not been for Mr. Flores' influence. At Stanford University, I benefited immensely from the seemingly infinite knowledge of Dan Fandrlick and Rajiv Dhawan. Both were very generous with their time as I learned the ropes as an undergrad in the Trost Lab, and they always maintained an upbeat attitude about research. Jason Woertink was also kind and helpful, both as a TA and as a research mentor in the Trost Lab. For my college years, I must also

thank Thierry Glauser, my fantastic internship supervisor at Guidant; his knowledge of polymers and stents and his humorous observations on life helped make for an excellent summer. Finally, during my senior year at Stanford, I had the good fortune of taking a course taught by Tom Wandless as well as completing research in his group. Without Tom's support, I would not have been able to pursue graduate research at Caltech, and I would not have discovered my keen interest in chemical biology.

Of course, the person who has had the largest impact on my thesis is my adviser, David Tirrell, and I am forever grateful that I had the opportunity to be part of his research group. The humility, patience, and humor which Dave exhibits are refreshing, especially at his level of professional success. I appreciate the freedom with which I was allowed to learn, develop, and pursue research in Dave's lab. Furthermore, his approach to mentorship and management have certainly had an impact on me as I begin thinking about my own career. I have no doubt that there are many aspects of Dave's role in my education that I have taken for granted and that will become more apparent as I progress through different stages of my career.

I am also so thankful for the support of my thesis committee members: Professors Peter Dervan, Julie Kornfield, and Sarah Reisman. The three of them have quite different research specialties, experiences, and personalities, and I learned a great deal from each of them. I appreciate their time and their efforts toward helping me grow as a scientist and as a person.

Next, I would like to express my gratitude to the members of the Tirrell Group who helped and supported me over the years. In particular, I am indebted to Rebecca Connor and John Ngo for teaching me nearly everything I know about bacterial protein

expression. Their patience, intelligence, and creativity were an inspiration to me. I greatly appreciate the scientific input and friendship of Stacey Maskarinec and Maren Buck, more than I can express in words. Conversations with Tae Hyeon Yoo, Jim Van Deventer, Julie Champion, and Janek Szychowski were helpful in shaping the direction of some of my thesis projects. Additionally, Frank Truong, Beverly Lu, Alborz Mahdavi, JD Bagert, Kai Yuet, and Brett Babin also provided scientific advice and ideas. In my last two years at Caltech, I had the pleasure of collaborating with Tamara Kinzer-Ursem, from whom I have learned a great deal about science and much more. Tami's infinite passion for research, even (and especially) when things are rough, is admirable. Thanks to Tami, I also had the chance to work alongside the scientists at Maven Biotechnologies and learn more about life and research at a small company. Finally, the summer of 2011 was particularly productive and fun due in part to the hard work and bright personality of Megan Lo, a talented SURF student whom I had the opportunity to mentor.

We have a terrific set of staff members here at Caltech, especially in the CCE Division, and I will miss many of them. The various administrators with whom I interacted—Anne Hormann, Agnes Tong, Karen Baumgartner, Laura Howe, Dian Buchness, and Laura Lutz King—put a superhuman amount of effort into keeping the labs, departments, and division running. I also want to thank Steve Gould, Leah Mentch, and Joe Drew for submitting and delivering my orders for the past six years; I really don't know how they keep track of all of our purchases and remember our names too! Lastly, Cora Carreido and Pat Perrone always did such a wonderful job keeping our stockrooms in shape and our experiments running. All of these folks not only do their jobs well, but

they do them with a smile, and I hope they know how much the students and post-docs appreciate their work.

The scientific facilities staff at Caltech were also instrumental to the research presented in my thesis. The mass spectrometry results critical to some of my projects were obtained with the aid of Mona Shahgoli, Robert Graham, and Jie Zhou. I greatly appreciate David VanderVelde's tireless efforts in maintaining and improving the NMR facilities, which were indispensable for characterizing many of the compounds which I worked with. Finally, Thai Truong and the staff at the Beckman Imaging Center were also helpful over the years.

The process of completing a Ph.D. thesis is made considerably more enjoyable by the support (and commiseration) of friends. I'd like to thank a number of other chemistry graduate students in the incoming class of 2006—Young In Oh, Jean Li, Pam Sontz, Natalie Muren, Hang Song, Nyssa Puskar, Heather Williamson, Charlotte Mason Whited, Narae Park, Chris Daeffler, Ian Tonks, Jacob Bitterman, Justin Chartron, and Patrick Theofanis—for countless lunch breaks, girls' nights, and problem set sessions, especially during our first few years. It was always nice to run into my classmates around campus in later years, too. In addition, I appreciate the camaraderie of so many other students and post-docs in the chemistry department; I'd especially like to acknowledge the Barton Group, who were very welcoming to me over the years, and many members of the Stoltz Group, particularly Jenny Roizen, who was an excellent TA and a dear friend.

I am also extremely grateful for the support, laughs, and perspective offered by my friends outside of Caltech. Conversations with Andrea Burbank, Julie Kim, David Lin, Grace Hsu, and Michelle Bailey Sidwell were integral to my maintaining my sanity

while at Caltech, especially at tough times. As graduate work can become all consuming, it was good and important to be reminded of the world beyond my lab bench, Spalding, and Caltech.

Another important factor in my maintaining some balance during graduate school was the set of student activities in which I participated. The Women Mentoring Women program was a fantastic outlet, offering opportunities to meet other women on campus and learn about navigating the scientific world. A huge thanks goes out to Portia Harris for the time and effort she pours into WMW and other student programming. I enjoyed serving as a mentor, mentee, and advisory board member for WMW over the past six years, and I'm excited to see what the future holds for the program. As I work with future generations of female scientists, I hope to implement what I learned from WMW. I also have no doubt that the closest friends I made through WMW—Kathleen Spencer and Kim Petersen—will continue to be a part of my life for many years to come. Finally, I loved playing flute in the Caltech Concert Band during my first few years of graduate school, and I will always cherish the memories of our magical trip to Carnegie Hall in 2008. It is thanks to William Bing that all of us in band were able to perform at that magnificent venue, and more importantly, were able to unwind and have fun while making music every week here in Pasadena.

I do not exaggerate when I state that I would not be receiving a Ph.D. were it not for my roommate and close friend, Chithra Krishnamurthy. She is like family to me now; no doubt, the most difficult part of transitioning to my post-Caltech life will be her physical absence, though it will be offset slightly by the certainty that we will be lifelong friends. I am so grateful for all the conversations we had about life and research over

meals in our apartment, for all that I learned from her, and for all the fun we had exploring LA when we finally did venture beyond lab and our apartment. She is truly the strongest, wisest, and most graceful person I have ever met. I also want to thank my honorary roommate, Keith Keitz, for his calm demeanor, sense of humor, and willingness to go along on the crazy exploits that Chithra and I planned for the four of us.

And this brings me to my best friend, my love, my husband, Eric Olmon. His infinite patience and steady support served as a solid foundation for me to lay my worries and troubles on every day, and his love for me is apparent in his every word and action. I am so, so thankful for all that he did for us during our time at Caltech, and I am super excited for what great adventures the future holds for us!

ABSTRACT

Proteins are involved in myriad processes in all organisms. They provide structural support in the membrane and scaffolding of each cell; they aid in the transmission of biochemical signals within and between cells; and they play central roles in combating various disease states. The development of techniques enabling selective and site-specific functionalization of proteins is an active area of investigation, as such modifications are critical to many studies and uses of proteins. For instance, with the addition of a unique reactive handle, a protein may be conjugated to a polymer for the production of protein-based therapeutics exhibiting improved bioavailability. Alternatively, proteins may be attached to slides to prepare diagnostic microarrays, reacted with hydrogels to create functional biomaterials, or decorated with fluorophores for *in vivo* imaging. Site-specific protein tagging techniques have already contributed greatly to biomedical research and will continue to advance the state of the field.

The focus of my thesis research has been the development of a novel site-specific protein labeling method centered on the eukaryotic enzyme N-myristoyl transferase (NMT). In a process called myristoylation, NMT appends a fatty acid to the N-terminus of numerous substrate proteins. Previous work demonstrated that NMT tolerates a wide range of chemically functionalized analogs of its natural fatty acid substrate. Here, we describe efforts that exploit various features of NMT: its ability to bind and utilize reactive fatty acid analogs, its exquisite selectivity toward its protein substrates, and its orthogonality toward those proteins naturally present in bacteria.

First, in Chapter II, we discuss the development of a model system for NMT-mediated protein labeling in the bacterium *Escherichia coli*. We synthesized an azide fatty acid analog that can participate in bioorthogonal chemistries, and we prepared two GFP-based substrate proteins, each displaying a recognition sequence derived from a known substrate of NMT. Our experiments indicate that labeling by NMT is site-specific, quantitative, and highly selective for each engineered substrate within the bacterial milieu.

As summarized in Chapter III, the model system was extended to the N-terminal labeling of two neuronal proteins, calcineurin (CaN) and calmodulin (CaM). While CaN is naturally myristoylated, CaM was engineered to achieve labeling by NMT. Experiments with CaN and CaM confirmed that our NMT-based system is quantitative and selective in its labeling of both natural and engineered substrate proteins. Extensive characterization of each protein allowed us to identify constructs that retain wild-type levels of activity even after labeling with the azide fatty acid.

Three of the protein constructs reported in Chapters II and III were utilized for microarray studies, as described in Chapter IV. We achieved rapid surface immobilization of each azide-labeled protein directly from lysate, a significant advantage when considering the time and resources normally required to purify proteins for downstream applications. The experiments and methods summarized in this chapter will be adapted for high-throughput biochemical research with protein microarrays.

Finally, the orthogonality of NMT toward bacterial systems was probed further for the purpose of selective labeling of individual bacterial proteins for live-cell imaging. In addition to identifying an azide fatty acid suitable for such studies, we also selected

two bacterial proteins that exhibit interesting functions and localization patterns, and we developed corresponding protein constructs for NMT-mediated labeling. Progress toward the use of NMT for *in vivo* imaging applications in bacteria is described in Chapter V.

Ultimately, our objective throughout the design and execution of these projects was to create and validate a new technique to achieve site-specific protein labeling. The particular advantages of NMT include its tolerance of reactive fatty acid analogs and engineered substrate proteins, and its lack of interaction with proteins present in the widely used *E. coli* expression host. We believe that the ideas and results presented in this thesis establish NMT-mediated protein labeling as a valuable tool for addition to the existing set of site-specific protein labeling methods. Development of such methods represents an important and exciting area within the field of modern chemical biology.

TABLE OF CONTENTS

Acknowledgements	iii
Abstract	ix
List of Figures, Schemes, Charts, and Tables	xv
CHAPTER I	I-1
<i>Protein Engineering: Techniques and Applications</i>	
Abstract.....	I-2
Protein Engineering	I-3
Incorporation of Non-Canonical Amino Acids into Proteins	I-3
Modification of the Protein N-Terminus	I-5
N-Myristoyl Transferase.....	I-6
Structure and Function of N-Myristoyl Transferase.....	I-6
Substitution of Fatty Acid Analogs for Myristic Acid.....	I-8
Thesis Organization	I-10
Overview of Thesis Chapters.....	I-10
Thesis Contributions	I-13
References.....	I-14
CHAPTER II	II-1
<i>Development and Evaluation of a Bacterial Model System for N-Terminal Protein Labeling with N-Myristoyl Transferase</i>	
Abstract.....	II-2
Introduction.....	II-3
Selection of 12-Azidododecanoic Acid (12-ADA).....	II-3
Two-Plasmid Bacterial Co-expression System.....	II-4
Results and Discussion	II-6
Synthesis and Characterization of 12-ADA.....	II-6

Expression and Purification of GFP-Based Substrate Proteins	II-7
MALDI-MS Analysis of Trypsinized Protein Samples.....	II-9
Intact LC-MS Analysis of Protein Samples.....	II-12
Fluorescence Gel Analysis of Lysate Samples	II-15
Conclusion	II-17
Experimental Section.....	II-17
References.....	II-22

CHAPTER III..... III-1

Selective N-Terminal Modification of Calcineurin and Calmodulin

Abstract.....	III-2
Introduction.....	III-3
Calcineurin and Calmodulin	III-3
Engineering CaM for NMT-Mediated Protein Labeling	III-5
Results and Discussion	III-7
Expression and Purification of CaN	III-7
Cloning, Expression, and Purification of Wild-Type and Engineered CaM Constructs	III-7
Intact LC-MS Analysis of Protein Samples.....	III-10
Evaluation of CaN and Engineered CaM Constructs via Phosphatase Activity Assays	III-13
Calcium-Binding Behavior of Engineered CaM Constructs	III-16
Fluorescence Gel Analysis of Lysate Samples	III-18
Conclusion	III-20
Experimental Section.....	III-20
References.....	III-27

CHAPTER IV..... IV-1

Surface Capture of N-Terminally Functionalized Proteins out of Lysate

Abstract.....	IV-2
Introduction.....	IV-3
Immobilization of Proteins on Surfaces.....	IV-3

Results and Discussion	IV-5
Experiments with Alkyne-Functionalized Agarose Beads	IV-5
Overview of LFIRE Instrumentation and Experimental Set-up	IV-11
Surface Capture and LFIRE Analysis of yARF-GFP Lysates.....	IV-14
Surface Capture and LFIRE Analysis of CaN Lysates.....	IV-16
Surface Capture and LFIRE Analysis of hCaNB-CaM Lysates.....	IV-19
Conclusion	IV-22
Experimental Section.....	IV-23
References.....	IV-27

CHAPTER V..... V-1

Progress toward the In Vivo Visualization of Individual Bacterial Proteins after N-Terminal Labeling

Abstract.....	V-2
Introduction.....	V-3
Organization and Localization of Bacterial Proteins.....	V-3
NMT-Mediated Protein Labeling for Imaging Studies in Bacteria.....	V-4
PyrG and MreB.....	V-5
Results and Discussion	V-7
Studies of Azide Fatty Acid Analogs: Dye-Labeling Lysate.....	V-7
Studies of Azide Fatty Acid Analogs: Dye-Labeling Live Cells.....	V-11
Preparation and Evaluation of yARF-PyrG and yARF-MreB Constructs.....	V-15
Conclusion	V-22
Experimental Section.....	V-23
References.....	V-27

CHAPTER VI..... VI-1

Concluding Remarks and Future Work

Summary of Thesis Work.....	VI-2
Further Studies.....	VI-6
References.....	VI-8

LIST OF FIGURES, SCHEMES, CHARTS, AND TABLES

Figure I-1. Non-canonical amino acid surrogates.....	I-3
Figure I-2. Process of myristoylation	I-7
Figure I-3. Crystal structure of NMT.....	I-8
Figure I-4. Overview of thesis projects	I-12
Chart II-1. Structures of myristic acid and 12-azidododecanoic acid (12-ADA)	II-4
Figure II-1. Overview of bacterial co-expression system.....	II-6
Scheme II-1. Synthesis of 12-azidododecanoic acid (12-ADA).....	II-7
Figure II-2. SDS-PAGE analysis of native Ni-NTA purification fractions for yARF-GFP and Fyn-GFP	II-9
Table II-1. Masses of yARF-GFP and Fyn-GFP N-terminal fragments.....	II-10
Figure II-3. MALDI mass spectra of yARF-GFP and Fyn-GFP.....	II-11
Table II-2. Masses of intact yARF-GFP and Fyn-GFP	II-13
Figure II-4. Intact mass spectra of Fyn-GFP purified from expressions in M9, LB, or 2xYT media	II-14
Figure II-5. SDS-PAGE analysis of yARF-GFP and Fyn-GFP lysate samples after TAMRA dye-labeling.....	II-16
Figure III-1. Crystal structures of CaN and CaM.....	III-4
Table III-1. Summary of engineered CaM constructs developed for N-terminal protein labeling studies	III-6
Figure III-2. SDS-PAGE analysis of phenyl sepharose purification fractions for CaM constructs.....	III-9
Table III-2. Intact LC-MS results for CaN and CaM constructs.....	III-11
Table III-3. Intact LC-MS results for hCaNB-CaM time course study.....	III-12
Figure III-3. [CaM]-dependent phosphatase activity assays.....	III-14

Figure III-4. Calcium-binding behavior of CaM constructs.....	III-17
Table III-4. Binding constants for phosphatase activity assay graphs	III-18
Figure III-5. SDS-PAGE analysis of CaN, hCaNB-CaM, and hCaNB-Linker-CaM lysate samples after TAMRA dye-labeling	III-19
Figure IV-1. Overview of functionalization of agarose beads.....	IV-6
Figure IV-2. Fluorescence of beads after incubation with dyed lysate, before and after washing	IV-8
Figure IV-3. Fluorescence of beads after reaction with azide-dye in absence or presence of lysate.....	IV-10
Figure IV-4. Overview of LFIRE instrument and LFIRE experiments.....	IV-13
Figure IV-5. LFIRE 3-D surface plots of microarrays after incubation with yARF-GFP lysate and related samples	IV-15
Figure IV-6. Average LFIRE signal for microarray spots after incubation with yARF-GFP lysate and related samples	IV-16
Figure IV-7. LFIRE 3-D surface plots of microarrays after incubation with CaN lysate samples	IV-17
Figure IV-8. Quantitative analysis of CaN surface plots: signal versus time and average post-wash signal.....	IV-19
Figure IV-9. LFIRE 3-D surface plots of microarrays after incubation with hCaNB-CaM lysate samples	IV-20
Figure IV-10. Quantitative analysis of hCaNB-CaM surface plots: signal versus time and average post-wash signal	IV-22
Figure V-1. Confocal microscope images of live bacterial cells after dye-labeling.....	V-4
Figure V-2. Crystal structures and localization patterns of PyrG and MreB	V-6
Chart V-1. Structures of azide fatty acids (azide FAs) and cyclooctyne-coumarin dye for <i>in vivo</i> labeling studies	V-8
Figure V-3. SDS-PAGE analysis of Fyn-GFP lysate samples with different azide FAs after TAMRA dye-labeling.....	V-9

Figure V-4. Fluorescence signal of <i>E. coli</i> cells after addition of an azide FA to growth culture and dye-labeling with cyclooctyne-coumarin	V-11
Figure V-5. Fluorescence signal of <i>E. coli</i> cells after addition of no FA, myristic acid, or an azide FA to growth culture and dye-labeling with cyclooctyne-coumarin.....	V-13
Figure V-6. Growth rates for expression cultures of yARF-PyrG and yARF-MreB	V-16
Figure V-7. SDS-PAGE analysis of yARF-PyrG lysate samples after TAMRA dye-labeling	V-19
Figure V-8. SDS-PAGE analysis of yARF-MreB lysate samples after TAMRA dye-labeling	V-21