Biophysics and Protein Engineering with Noncanonical Amino Acids

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

2011

(Defended April 19, 2011)

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For My Family

ACKNOWLEDGEMENTS

I am grateful to numerous individuals for their help and support during the pursuit of my doctoral degree. First, I would like to thank my advisor, Professor David Tirrell, for providing such comprehensive support, financial and otherwise, during my time in his laboratory. He presented me with an environment in which I was free to boldly pursue problems at the interface of chemistry and biology. I am extremely thankful for Dave's rigorous approach to scientific analysis, as his approach has trained me to be a very careful scientist. Moreover, the scientific freedom I have had during my time in the Tirrell laboratory has allowed me to develop an interest and passion for science and engineering that will continue to inspire me for years to come.

Many other members of the academic community have been very supportive of my work. My thesis committee, Professors Frances Arnold, William Clemons, and Mark E. Davis, have all provided helpful suggestions during my studies. I would especially like to thank Professor Arnold for helping me to understand some of the intricacies of natural evolution, directed evolution, and high-throughput screening. Professor Anand Asthagiri provided helpful comments as a member of my candidacy committee, and Professor Jim Swartz of Stanford University has been very generous with his time and advice over the past few years. Last, I would like to thank my undergraduate research advisor, Professor Stacey Bent, and her former student, Professor Michael Filler, for providing me with my earliest research opportunities as an undergraduate student at Stanford. Both Stacey and Mike were hugely supportive of me from the moment I started research, and I am extremely grateful for their encouragement to pursue a career in scientific research.

I have had the opportunity to collaborate with a number of colleagues during the course of my work. I thank Professor Ahmed Zewail and his laboratory members, Dr. Oh-Hoon Kwon and Dr. Christina Othon, for the pleasure of studying hydration dynamics with them. Collaborations with a number of former and current Tirrell lab members have also been extremely enjoyable over the years. Dr. Tae Hyeon Yoo was responsible for starting the hydration dynamics work with the Zewail Laboratory and provided support during its completion. He was also instrumental in training and advising me during early flow cytometry experiments, library constructions, and cell strain development. Professor A. James Link helped me perform enzymatic assays with radioactive chemicals and taught me some of the basics of molecular biology in support of studies on aliphatic ketone-containing amino acids. Professor Nick Fisk provided large-scale syntheses of homoisoleucine and other large, aliphatic noncanonical amino acids for use in biophysical studies. Professors Yi Tang and Pin Wang provided helpful comments during the completion of a manuscript on aliphatic ketone-containing amino acids. Kai P. Yuet has been instrumental during the completion of the antibody fragment engineering work. His enthusiasm, diligence, and attention to detail during the expression and characterization of more than thirty antibody fragments have been invaluable. It has been a pleasure to work with him during these efforts.

Life at Caltech has been very enjoyable thanks to the people I have been lucky enough to be surrounded with during my stay at the Institute. The Tirrell laboratory has been a wonderful place to work over the years, and I have appreciated conversing and interacting with so many thoughtful and kind scientists during my time in the lab. I especially thank Alborz, Alison, Beverly, Brad, Brett, Caglar, Chethana, Eileen, Frank,

Inchan, Jamie, Janek, J. D., Jeremiah, Jin, John, Julie Champion, Julie Liu, Kai, Kechun, Kevin, Kimberly, Larry, Maren, Nick Ball, Nick Fisk, Phoebe, Rebecca, Shelly, Soojin, Stacey, Tae Hyeon, and Yvette for conversations about life and the lab. My chemical engineering classmates are another group of people that has made life more fun over the years. I especially thank Chase Beisel for many enjoyable conversations about science and science politics and Jason Gamba for discussions of science and sports over lunch. I also thank my friends from outside chemical engineering for their help and encouragement during my scientific studies. These include Alberto Cueto, Katie and Ted Brenner, and countless additional friends I have made through the church communities of Warehouse and Westside Christian Fellowship.

My final thanks go out to my family for their unending support and love. My parents Allen and Debbie Van Deventer have inspired and encouraged me at every step. My brother Paul has also been a great backer of my studies throughout my time at Caltech. I am so fortunate to have had them all so close by while working on my degree, and their presence has enriched my life greatly over the past few years. Last, I thank my wife Allison for being a steadfast companion to me ever since we started dating. I am so lucky to have found an amazing woman to spend the rest of my life with during my time in Pasadena. Her advocacy and encouragement have been invaluable in helping me to persist and persevere through all of the challenges of completing a doctoral degree at Caltech. I am a much better person and scientist today thanks to her constant love and loyalty.

ABSTRACT

Noncanonical amino acids are tools for expanding and altering the chemical functionalities available within proteins. Much recent work has focused on developing biosynthetic means for incorporating noncanonical amino acids into proteins, and applications of noncanonical amino acids to many problems in science and engineering are emerging. The first portion of this thesis describes established methods to incorporate noncanonical amino acids into proteins and efforts to exploit the properties of noncanonical amino acids in areas such as protein structure determination, protein and organism evolution, modulation of the immune system, and proteomics. Researchers' creative and successful use of this growing toolkit suggests that noncanonical amino acids will continue to be a valuable asset for dissecting biological problems and imparting proteins with new chemical and physical properties.

Biophysical studies with noncanonical amino acids provide a platform for studying the effects of atom-by-atom manipulations of amino acid side chains on protein properties. The middle portions of this thesis describe work to better understand how protein properties are affected by subtle amino acid side chain manipulations. This work was aided greatly by the establishment of homoisoleucine as a translationally active analog of leucine in bacterial cells. The small side chain differences between leucine, homoisoleucine, and the fluorinated amino acid trifluoroleucine allow for detailed studies on how amino acid side chain size and fluorination affect protein stability and hydration dynamics. Replacement of leucine by homoisoleucine in coiled-coil peptides stabilizes these proteins, as shown by elevation of the coiled coil thermal denaturation temperature. The stabilization observed when homoisoleucine replaces leucine in the peptides is greater than when

trifluoroleucine replaces leucine, suggesting that expansion of side chain volume may play a role in protein stabilization irrespective of hydrocarbon or fluorocarbon character.

Studies of water-protein interactions using designed coiled coils containing surface-exposed leucine, homoisoleucine, or trifluoroleucine residues enabled systematic examination of the roles that side chain size and fluorination play in dictating solvation dynamics. Fluorinated side chains appear to exert a large electrostatic drag on nearby water molecules. These results have important implications for the design and engineering of fluorinated proteins due to the critical role water-protein interactions play in many protein properties and functions.

The final portion of this thesis details efforts to engineer the binding properties and chemical reactivity of antibody fragments with noncanonical amino acids. The properties of the single chain variable fragment form of a model anti-digoxin antibody have been studied after replacement of the protein's methionine residues with methionine analogs containing alkyne, azide, and aliphatic side chains. Experiments with antibody fragments displayed on the surface of *Escherichia coli* cells revealed that replacement of the methionine residues of the fragment with an analog containing an alkyne side chain reduced the fluorescence levels of cells treated with a fluorescently labeled antigen to background levels, indicating loss of binding function. Replacement of methionine with analogs containing aliphatic and azide side chains left the fluorescence of cells unchanged and reduced by a factor of 0.6, respectively. Fluorescence-activated cell sorting of libraries of cell surface-displayed antibody fragments enabled the isolation of clones functional in multiple amino acid contexts. Cells displaying variants containing alkyne, azide, and aliphatic analogs and treated with fluorescently labeled antigen were more fluorescent than

cells displaying the methionine form of the parent antibody fragment by factors of roughly 1.7, 3.5, and 1.3, respectively. Furthermore, the amino acid context used during high-throughput screening experiments appears to affect the frequencies of mutations occurring at various positions within the antibody fragment construct. High-throughput sequencing revealed that populations isolated in different amino acid contexts exhibit mutational rates differing by greater than twenty percent at some residues in the protein.

Characterization of soluble fragments indicated that each noncanonical amino acid used in this study modulates the binding kinetics of antibody fragments in a distinct fashion. Perhaps most interestingly, fragments containing the azide-containing analog azidohomoalanine exhibit improved binding kinetics relative to their methionine-containing counterparts. Replacement of methionine by azidohomoalanine in several variants lowers the dissociation constant of the fragment by up to a factor of two. Chemical conjugation of azide-containing fragments to fluorescent dyes and biotin proved facile with strain-promoted cycloaddition reactions. Quantifications of the extent of reaction using fluorescent dyes revealed that approximately 0.4 dyes had been conjugated per protein, and the resulting conjugates were found to retain their binding function in kinetic and Western blotting assays. Experiments in which azide-containing fragments were displayed on the surface of *Escherichia coli* cells and subjected to strain-promoted cycloadditions demonstrated that the extent of chemical modification and antigen binding can be monitored simultaneously and used to isolate cells displaying functional, modified proteins. These experiments demonstrate how noncanonical amino acids can be used to modulate multiple properties of antibody fragments and illustrate the feasibility of developing and

screening libraries of chemically modified proteins. Evolved, functional bioconjugates may be applicable to a variety of outstanding diagnostic and therapeutic problems.

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