Site-specific incorporation of synthetic amino acids into functioning ion channels

Thesis by

Gabriel Shaw Brandt

In Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

2003

(Defended October 4, 2002)

in memory of Teresa Hsu and Norman Davidson

Acknowledgments

The life of a man is a dubious experiment.
C. G. Jung

First of all, I'd like to put in a good word for our nation's high school science teachers, who in my case were Linda Bell, Mark Smith, Gary Miller, and Ron McCray. Good teachers make the difference all the way along, and I've benefited from an excellent crop at Reed College and here at Caltech. Dennis is an excellent advisor, a deeply humane individual who invariably reaches for the carrot rather than the stick. He's been a fount of scientific clarity and sound advice from my very first days at Caltech. Henry is a voracious collaborator, who's allowed a generation of Dougherty grad students to travel where chemists usually fear to tread. Through his guidance, along with that of Norman Davidson, I've been able to transcend disciplinary boundaries a little and try to address real biological questions. Doug Rees, John Bercaw, Peter Dervan, and Barbara Imperiali, the past and present members of my committee, have been excellent as teachers and periodic mentors.

Grad school becomes a lifestyle after a while, so it's difficult to distinguish among friends, colleagues, associates, mentors, accomplices. Rather than just jumbling everyone together, I'll divide it up this way. These are people associated with our labs to whom I would owe a debt of gratitude, if I didn't consider most of them to be good friends: Alison McCurdy, for her wise ways; Purnima Deshpande, Cesar Labarca, Mark Nowak, Paolo Kofuji, and Craig Doupnik of the Lester labs, who helped initiate me into the world of ion channels and the frog eggs who love them; Pam England, whose friendship, creativity, and wide-ranging curiosity got me started off right; Jesse Lin, Sarah Ngola, Seth Miller, and Jennifer Ma, whose openness, on-the-ballness, and general

coolness helped persuade me to join this lab; Scott Silverman, Anthony West, and Wenge Zhong, for all the former plus their scientific assistance with all aspects of unnatural amino acid mutagenesis; Yanhe Tong, for identifying a good idea when he saw it, and for sticking it out when things got ugly; Ken Philipson, for his generosity of spirit; Justin Gallivan, for oodles of scientific advice over the years; Marcus Sarofim, for any manner of assistance selflessly proffered; Lintong Li, for her inspiring commitment to learning at every opportunity, whether experimental or linguistic; Bal Khakh, for his enthusiasm for all things purinergic; Don Elmore, for leavening our drab existence with poetry and Midwestern charm; David Dahan, for impeccable scientific logic and admirable devotion to cats; James Petersson, for congenial camaraderie, conversation, and chemical commiseration as needed; Sarah Monahan, who's always ready for a conversation about psychoneuroimmunopharmacology; Darren Beene, for following the True Path; Steve Spronk, whose commitment to the positive is positively infectious; Lisa Turner, whose perspicacity and tenacity were continually tested by the wickedness of receptors; Anita Choi, for her efforts to make everything work out in the end; and to Tingwei Mu, Amanda Cashin, Michael Torrice, and Jordan Benjamin, who have every intention, I'm sure, of rocking on. In a category by themselves are Josh Maurer and Niki Zacharias. Josh probably does himself a disservice by being too helpful. The barrier to figuring something out myself is usually far higher than the barrier to bugging Josh to find out how to do it. Plus, he's a josh-of-all-trades, so practically everything I've done as a grad student has been with his assistance. It's hard to estimate the amount of time, all of it enjoyable, that I've spent with Niki, my friend, colleague, and housemate. My experience here has been inestimably improved by sharing it with her. Insofar as

perseverance and generosity are what makes the world a better place, she's certainly doing her part.

Next on this list, but in no way secondary, are friends who happened not to be in my lab. Mike Johnson took me under his wing when I first arrived in Pasadena. Megan Nuñez and Ben Lane, along with Marcus, welcomed me into their house. Liz Boon and Isaac Carrico have been wonderful housemates and friends. Jason Belitsky provided the example for thesis writing with an impressive closing effort. Michael and Traci Shogren-Knaak have been steadfast companions and have been keeping my life together for years, both scientifically and psychologically. My dear friend Catherine Baker has been an evergreen source of lexicology, phytophilia, racing tips, stories, and general wit and warmth.

Others at Caltech who have been crucial for making the experience tolerable, even enjoyable at times, are the many friends with whom I've played soccer, Frisbee, or music, along with Bill and Delores Bing, Donald Caldwell, and Allen Gross.

Finally, here are select friends outside of Caltech: Kris McNeill and Shana Sturla, Jeff and Saralyn Ang-Olson, Julie Veltman, and Christi Corbett have all improved my quality of life without even being here. Most of all, I'm grateful to my beloved friend Pamela Surkan for waiting for me and for introducing me to peixe-boi filhotes.

Caltech seems to have a knack for hiring incredibly competent and friendly people, from whom I've received a great deal of assistance, particularly Dian Buchness, Chris Smith, Steve Gould, Debbie Miles, Linda Syme, Shannan Boss, and Vanna Santoro.

Lastly, special thanks to my parents for not listening to that Willie Nelson song about letting your kids grow up to be chemists.

Abstract

The ability to introduce a synthetic amino acid into a fully folded protein allows the full power of organic chemistry to be applied to protein biochemistry. Any functionalized amino acid which can be incorporated by the ribosomal machinery may be site-specifically introduced. Through the application of chemical creativity, mimicry of the natural behavior of protein side chains and the introduction of novel function may both be attained.

As a class, integral membrane proteins require advanced biochemical tools for their characterization, since many of the classical methods of biochemistry are not applicable. These molecules represent an opportunity for the acquisition of unique information through the use of unnatural amino acid mutagenesis.

Cell-cell communication is fundamental to neurobiology, and leads ultimately to the phenomenon of consciousness. The receipt of extracellular stimuli relies on integral membrane proteins, and membrane-bound ion channels and receptors are the central proteins of molecular biology. Just as integral membrane proteins are well-suited to investigation by unnatural amino acid mutagenesis, molecular neurobiology is an excellent area for the application of this technology.

In the work presented here, tools for the measurement of physical organic parameters associated with molecular recognition events and conformational changes of proteins are developed and implemented in functioning neuroreceptors. In addition, analytical tools are introduced and deployed to investigate the real-time modulation of ion channel function in living cells.

After the work is introduced in Chapter 1, experiments on the use of fluorinated tryptophan analogs to serially modulate the electrostatics of particular amino acid side chains are presented in Chapter 2. The goal of these investigations is to understand an interaction between nicotine and tryptophan residues in the nicotinic acetylcholine receptor. Nicotine is shown not to experience a cation-π interaction with the side chain that mediates this interaction between the receptor and its natural agonist, acetylcholine. Additional studies on analogs of both nicotine and acetylcholine are presented, along with attempts to extend the fluorinated tryptophan methodology to neuronal receptors of the same class.

A series of dynamic amino acids are presented in Chapter 3. The overarching goal of these studies is to obtain information on ion channel conformation, both *in situ* and subsequent to isolation. A photoactive amino acid which induces proteolysis at the site of its incorporation is shown to have significant effects on the nicotinic acetylcholine receptor. Efforts to extend this methodology to biochemically detect backbone cleavage in the functionally affected receptors are also presented in this chapter. Also, the hydrolysis of an ester linkage introduced by the incorporation of a hydroxy acid in place of a natural amino acid is attempted to identify the disulfide connectivity of rat $P2X_2$ receptors. Finally, attempts to utilize photoreactive amino acid side chains to both crosslink adjacent subunits of the nicotinic acetylcholine receptor and to induce local conformational perturbations in the transmembrane regions of this receptor are detailed.

In Chapter 4, tyrosine containing a photo-removable protecting group is introduced in place of a particular tyrosine residue on which the modulation of Kir2.1 channel function depends. By this means, experimental control is gained over the chemical identity of this

side chain. As introduced, it is neither a substrate for tyrosine kinases nor for protein-protein interactions. However, once photolysis has revealed the wild-type residue, these interactions may occur. Co-injection of the tyrosine kinase v-Src along with the irradiation of cells expressing Kir2.1 containing this caged tyrosine residue at position 242 produces a 50% current reduction over a time course of approximately ten minutes. The roles of phosphorylation and endocytosis in causing this reduction were extensively investigated.

The final chapter presents progress toward controlling the *in situ* phosphorylation state of particular residues in a protein. A general method for synthesis of caged phosphoamino acids is developed and applied to the synthesis of analogs of serine, threonine, and tyrosine. A variety of routes toward caged non-hydrolyzable phosphoamino acid analogs are shown, along with the preparation of important synthetic intermediates.

Table of Contents

List of figures

List of tables