Chapter 1. Unnatural amino acid mutagenesis in molecular neurobiology

1.1 The nature of the interface between chemistry and biology

The smallest meaningful unit of a biological system is probably the molecule. Chemists working in the life sciences thus represent the reductionist extreme of biology. Interestingly, biological molecules appear enormous and complex to chemists, and are closer to the holistic end of our intellectual spectrum. The individual biochemist, then, seeks to publish in an environment where both reductionist and holistic thinking count. The general tenor of biological investigation is less reductionist than our usual work, and the viewpoint of most chemists is more so. Thus, the challenge of biochemistry is often pinpointing the specific interactions that interest us as chemists and yet putting them in the perspective of their effect on organisms as a whole. This increased level of generality in biological chemistry often arises through method development. To take a well-known example, the 1991 Nobel Prize in chemistry rewarded the development of two general methods, site-directed mutagenesis and PCR, which have driven much of the biology of the last two decades.

The work presented in this thesis focuses in detail on a number of different proteins and a variety of scientific questions. Following the strategy suggested above, the common theme is a technique. The general method that unites the disparate elements of this thesis is unnatural amino acid mutagenesis, performed on integral membrane proteins, in living cells.

1.2 Nonsense suppression and molecular neurobiology – a felicitous combination

Actually, there is a common intellectual theme as well. All of the questions addressed in this work, though they may differ from the perspective of biochemistry, are questions about neuroreceptors. Molecular neurobiology is a science which has arisen in the latter half of the 20th century. The seminal predictions of Hodgkin, Huxley, and Katz that ion permeability was responsible for the unique behavior of neurons and that dynamic ion channels must account for electronic excitability, action potentials, and the propagation of nerve signals were made largely from 1930-50. In the latter decades of the century, the molecules responsible for neural excitability began to be characterized at the atomic level.

Ion channels and neuroreceptors are molecules like any other, and the full range of techniques of molecular biology and biochemistry is available for their study. They are integral membrane proteins, however, and are thus refractory to many of the manipulations available to soluble proteins. However, they have a remarkable property unavailable to other biological molecules. Unique to these membrane-bound receptors is the ability to regulate the flow of ions across cellular membranes. This ion flux can be very readily measured, and physiologists have developed sophisticated methodology for studying this phenomenon. In addition to making up in some ways for their challenging biochemistry, the electrophysiological response of neuroreceptors is a very sensitive technique. Chemists are generally unused to being able to measure single-molecule events, but the patch clamp permits the electrophysiology of single channels to be measured. If electrophysiology provides a sensitive means to detect the activity of ion

channels, unnatural amino acid mutagenesis provides a sensitive method for manipulating them.

The introduction of unnatural amino acids into proteins may be accomplished by nonsense suppression.[1-5] The particular codon in a gene which encodes the amino acid to be replaced is changed to a non-coding, or 'nonsense' codon. Typically, this is a stop codon. The conversion of a coding codon to a stop codon normally induces termination of translation and a protein truncated at the position of stop codon introduction. If, however, the cell has also been supplied with tRNA molecules modified to recognize this stop codon and carrying an amino acid at their 3' end, translation continues apace. The suppression of the phenotype arising from the presence of a truncated protein in the cell, caused by a nonsense codon, is what gives this phenomenon its name. Nonsense suppression may easily be achieved in an organism by the simple molecular biological manipulation of mRNA and tRNA.

Nonsense suppression is used to introduce unnatural amino acids by charging the suppressor tRNA with a synthetic amino acid. It has been observed that the ribosome, which is responsible for translating mRNA into proteins, is rather tolerant in its accommodation of amino acid side chains. In addition to the natural 21 amino acids which are genetically encoded, the ribosome will accept quite a large number of unnatural amino acids. Thus, if a cell can be provided with two materials, an mRNA altered to contain a stop codon and a tRNA engineered to recognize the stop codon and carrying a synthetic amino acid at its 3'end, it is possible to introduce an unnatural amino acid into a protein by nonsense suppression. (Figure 1.1) In this work, the nomenclature

of nonsense suppression and unnatural amino acid mutagenesis will be used interchangeably.

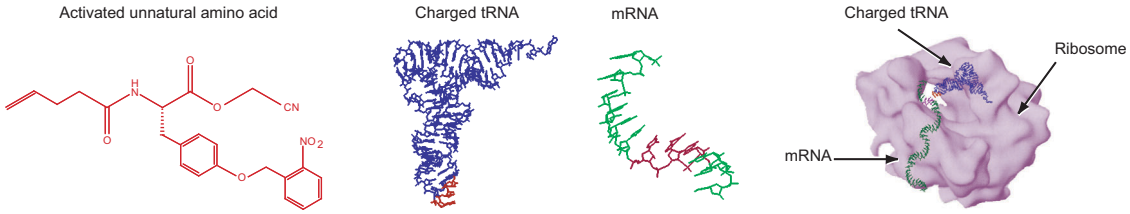

Figure 1.1 Schematic of the components necessary for unnatural amino acid mutagenesis. Left: Unnatural amino acid, C-terminally activated as the cyanomethyl ester for chemical conjugation to tRNA, and N-terminally protected for stability. Center: Suppressor tRNA (anticodon indicated in red) and mRNA containing a stop codon (also red) at the site of desired synthetic amino acid insertion. Right: Ribosomal translation, using the synthetically aminoacylated tRNA (blue) and stop codon-containing mRNA (green) to generate a protein containing an unnatuaral amino acid.

The marriage of ion channel electrophysiology and unnatural amino acid mutagenesis is consummated in the cells of a South African clawed frog. The oocyte of *Xenopus laevis* has been discovered to be an excellent vehicle for the functional expression of a wide variety of molecules fundamental to molecular neurobiology.[6] (Figure 1.2)

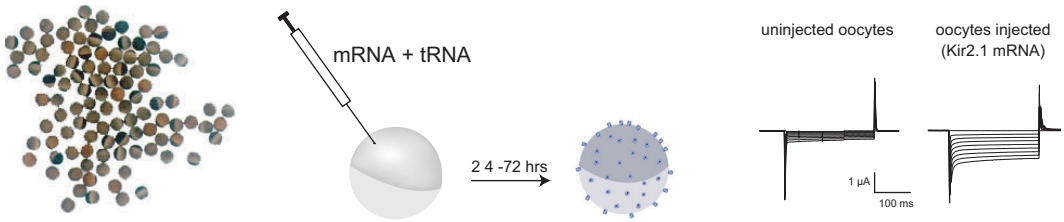

Figure 1.2 *Xenopus* oocytes are utilized in molecular neurobiology for the heterologous expression of ion channel proteins. Left: *Xenopus* oocytes at 1x magnification. Center: Schematic showing the microinjection of mRNA and tRNA into oocytes, followed by the expression of receptors (not to scale) at the cell surface. Right: Example of electrophysiological data obtained by two-electrode voltage clamp from oocytes expressing a potassium channel Kir2.1 arising from nonsense suppression.

Physical injection of mRNA into oocytes (these are very large cells, approximately 1 mm in diameter, which is why they were selected for this purpose) usually results in the expression of functional proteins. In addition, it was demonstrated in our laboratory in 1995 that these cells may accommodate also the co-injection of synthetically charged

suppressor tRNA, which results in the plasma membrane expression of ion channels containing synthetic amino acids.[7] Thus, in living *Xenopus* oocytes, unnatural amino acids are introduced site-specifically into functioning ion channels.[7-10] The behavior of these channels may be measured using electrophysiology.

1.3 Implementation of the suppression method in molecular neurobiology

1.3.1 tRNA synthesis

The organic chemistry required to synthesize amino acids is clearly well-established. However, the requirement to specifically attach a synthetic amino acid to a full-length tRNA is potentially a much bigger synthetic challenge.[11] A fundamental step in the development of the unnatural amino acid mutagenesis methodology was the realization that this synthesis may be largely avoided.[12] The bulk of the tRNA may be prepared using molecular biology. Only a small piece is chemically synthesized, the dinucleotide corresponding to the 3' end of the tRNA. The desired amino acid is chemically coupled to this dCA dinucleotide (the cytosine 2' hydroxyl is not necessary for translation, so dC is used instead of C for ease of specific aminoacylation). Then, the dCA-amino acid is attached to body of the tRNA by enzymatic ligation. The tRNA body itself is prepared by runoff transcription from a plasmid template, a very standard molecular biology procedure.

1.3.2 General considerations

1.3.2.1 Variability in suppression efficiency

The choice of which residue to replace clearly depends on the experiment. This is sometimes complicated by the fact that suppression efficiency is also highly variable from residue to residue. Some amino acids may not be readily replaced, or may be replaceable by some unnatural side chains but not others. In practice, we have identified no reliable guidelines for predicting which sites will work well. There is no substitute for simply attempting incorporation of the desired amino acid at the desired position. Fortunately, trying several different sites is a matter of fairly routine mutagenesis, so trial and error is not terribly time-consuming. There are a number of factors which may account for the observed variability in suppression efficiency.

1.3.2.2 Optimizing translational efficiency

Factors associated with translation include codon context, read-through of the stop codon, and effects from truncated proteins. First, the 5' and 3' nucleotide sequence around a stop codon can govern the efficiency with which it is suppressed by suppressor tRNA's.[13-16] Since some amino acids may be coded for by codons in unfavorable contexts, suppression at these positions may be difficult. It should be mentioned, however, that we have never observed a clear correlation between suppression efficiency and codon context, despite the large body of literature on the subject. It could be that trends are confounded by post-translational effects of incorporating unnatural side chains, some of which are discussed below. In any case, an unfavorable codon context can sometimes be ameliorated via silent mutations around the desired site. Codon bias,

where a heterologous expression system may translate mRNA poorly because their native codons represent rare tRNA's in the heterologous system, may be addressed similarly.[17-20]

A second difficulty which may arise at the translational level is read-through of the stop codon.[21] We use the term generally here to mean any process by which full-length protein is produced which does not contain the desired unnatural amino acid at the appropriate position. This may occur if a) the stop codon is recognized by an endogenous tRNA even without a perfect codon:anticodon match, or b) if the exogenously applied tRNA loses its unnatural amino acid and an endogenous synthetase replaces it with a natural amino acid, or c) if the anticodon is skipped to generate a mutant protein with a deletion at the desired incorporation site. Again, it has proven difficult to generate formal rules for predicting the likelihood of these events. Typically, the amount of injected mRNA or, if applicable, the ratio of subunits to each other can be altered to reach a regime where there is reasonable expression with little evidence of read-through.

Finally, suppression efficiency is far from quantitative, so translation frequently terminates at the introduced stop codon. When this occurs, the truncated protein which forms must be checked for its ability to function, any deleterious effect on cell health, or possible dominant negative effects.[22,23] Particularly with suppression near the C-terminus of polytopic membrane proteins such as receptors, partial-length proteins may be trafficked to the surface and interfere with certain experiments. Again, the best experimental recourse might be to adjust the amounts of mRNA involved.

1.3.2.3 Efficiency of folding and assembly

A second class of complications has to do with folding, assembly, and trafficking of proteins containing mutant side chains subsequent to translation.[24-26] Once again, it is

difficult to predict the side chains and sites at which this will be problematic. Also, it can be difficult to distinguish between improper folding and loss of function as a result of unnatural amino acid incorporation. These two scenarios, and also whether a problem is translational or post-translational, may sometimes be distinguished by SDS-PAGE. Separation of cytosolic from surface proteins followed by PAGE will reveal a) whether or not full-length protein is being produced by the cell, and b) whether or not the receptor is making it to the cell surface.

1.3.3 Essential controls

As a result of the translation and post-translational complications associated with the nonsense suppression methodology, it is absolutely essential to perform the requisite controls. The incorporation of caged side chains has the benefit of conferring chemical reactivity unavailable to the twenty naturally occurring amino acids, so it is usually straightforward to distinguish between proteins containing caged amino acids and those which do not. Nonetheless, the following controls are necessary each time suppression is attempted in a new receptor, at a new residue, or with a new amino acid analog.

1.3.3.1 mRNA only

Injection of the stop codon-containing mRNA without exogenous tRNA is a simple experiment which controls for a number of outcomes. If truncated protein is functional, this control should reveal it. Likewise, if read-through of the stop codon is occurring, it will be evident. The mRNA-only control with ion channels in *Xenopus* oocytes frequently gives rise to measurable currents, for instance. If a dominant negative effect from truncated protein is suspected, it is again a simple matter to co-inject mutant mRNA

with wild-type mRNA to see if the presence of the mutant RNA decreases the expression of functional protein.

1.3.3.2 mRNA with uncharged suppressor tRNA

Even if read-through of the mRNA alone is not occurring, the presence of uncharged exogenous tRNA frequently leads to full-length protein. Presumably, the exogenous tRNA is being recognized by endogenous synthetases or the suppressor tRNA facilitates skipping of the stop codon to generate a deletion mutant. Again, this problem can be addressed by decreasing the amount of mRNA injected into the cell to find a concentration which gives reasonable expression without measurable read-through. In this control, mutant mRNA is injected with 74mer tRNA enzymatically ligated to dCA. Alternatively, mutant mRNA is injected with 74mer tRNA. Eukaryotic cells transcribe tRNA in a truncated form and enzymatically add the final CCA bases to the 3' end. In oocytes, the 74mer often gives rise to greater read-through currents than full-length tRNA generated by ligating dCA to the 74mer. This confirms that oocytes are capable of completing truncated tRNA and suggests that perhaps dC at position 75 is a poorer substrate for endogenous synthetases than C, or that interaction with the ribosome is weaker, resulting in less full-length protein being produced.

1.4 Uses of unnatural amino acid suppression in molecular neurobiology

1.4.1 Structure-function studies

Because of the site-specificity of unnatural amino acid mutagenesis, it is an excellent tool for determining the functional role and structural context of particular side chains.

Since the range of amino acid side chains tolerated by the ribosome is rather generous, a number of functional and structural probes may be introduced.[1-5]

Chapter 2 presents an example of such a use of unnatural amino acids. This work follows the initial observation by Wenge Zhong that nicotine appears to bind the nicotinic acetylcholine receptor (nAChR) differently from either its natural ligand acetylcholine (ACh) or the weak agonist tetramethylammonium (TMA).[27] The chapter is largely based on a *Biochemistry* paper written in collaboration with Darren Beene, who performed an enormous amount of work on the neurotransmitter serotonin (5-hydroxytryptamine, or 5-HT) and its receptor, 5-HT_{3A}R.[28] Valuable synthetic assistance was also provided by Niki Zacharias, who prepared an *N*-alkylated analog of nicotine used in these studies. The second section of this chapter describes attempts to extend the use of our structure-function probe to a class of nAChR found in the central nervous system (CNS). Much of this work was carried out with great persistence and skill by Lisa Turner, during the summers of 1999 and 2000.

1.4.2 Dynamic in situ manipulation of ion channels

In addition to their use as probes, unnatural amino acids may be used to exert control over biological molecules while they are functional and in their native environment. This ability is actually almost without precedent, and could conceivably be used to carry out experiments which have no counterpart outside the realm of unnatural amino acid mutagenesis. Thus, there is undoubtedly information which could be supplied by these techniques which would be of great interest to the biological community. However, experiments of this nature remain technically challenging, as can be seen in Chapters 3-5.

1.4.2.1 Conformational studies

In Chapter 3, a number of experimental approaches to understanding details of the conformation of ion channels are presented. The first deals with attempts to site-specifically cleave the backbone of a functioning ion channel. This work is based on imaginative chemistry developed by Dr. Pam England in this laboratory in 1997.[29] The experiments presented herein were carried out with a great deal of useful assistance from her, and many of the ideas were developed with her guidance. The second area treated in this chapter is site-specific photocrosslinking. This work mirrors conventional photoaffinity labeling with the added benefit that the label may be introduced site-specifically. The experiments described in the section were carried out in collaboration with Justin Gallivan, who prepared the unnatural amino acid used in the studies and performed some of the electrophysiology.[30] Thirdly, a method for determining disulfide connectivity in folded, native proteins is presented. This section is also based on a method developed by Pam England.[31] Experiments to determine the cysteine topology of the ATP receptor $P2X_2$ were carried out with intellectual input from her and both intellectual and material collaboration with the knowledgeable and hard-working Dr. Baljit Khakh. Finally, a technique for dynamically altering the local conformation of a native protein the vicinity of a particular residue is presented. This work was carried out almost entirely by Dr. Ken Philipson, who generously shared authorship on a paper in the *American Journal of Physiology* for my assistance with the early phases of the project.[32]

1.4.2.2 Controlling protein-protein interaction and post-translational modification

The ability to introduce photoreactive amino acid side chains permits some control over the chemical identity of particular residues. Prior to photolysis, one chemical

species is present, while after photoinduced reaction an altogether different compound may be present. In Chapter 4, the photochemical conversion of a tyrosine analog to tyrosine itself is presented as a means of controlling interactions involving a particular Tyr residue. This chapter is based in its entirety on a paper in the *Journal of General Physiology* written in collaboration with Dr. Yanhe Tong, whose dedication in the face of an extremely difficult and complex problem was necessary to shepherd the project to a favorable conclusion.[33] He was responsible for all of the electrophysiology reported in this chapter, with the exception of the single-channel recordings, which were performed by George Shapovalov. Fluorescence assays were carried out with the able assistance of Dr. Ming Li. The introduction includes portions of an invited review in *Methods in Enzymology*.[34] The text is my own, written under the guidance of James Petersson and with additional editorial assistance from Niki Zacharias.

In Chapter 5, a similar strategy of photoreaction is introduced as a means of controlling phosphorylation, arguably the single most important biological regulatory pathway acting at the protein level. Where the work of Chapter 4 is based on revealing wild-type tyrosine, the experiments in this chapter represent progress toward developing a technique to reveal phosphoamino acids and their non-hydrolyzable analogs. The synthesis of these compounds was performed with the valuable help of Anita Choi, who prepared caged threonine and the important synthetic intermediate nitrobenzyl bromodifluorophosphonate, during the summer of 2002. Caged serine and threonine, mentioned in this chapter, were synthesized earlier in this laboratory by Justin Gallivan.[30]

1.5 References

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