Chapter 1

Introduction

1.1 The nonsense suppression methodology for unnatural amino acid incorporation

Site-specific incorporation of unnatural amino acids into proteins has become an invaluable tool in protein structure function studies. Examples include studies of protein stability, enzyme mechanisms, the incorporation of biophysical probes (fluorescent amino acids and spin labels) and photoreactive side chains (caged amino acids), and backbone mutations [1-5]. This technique was first employed by the use of in vitro translation systems (*E. coli* and rabbit reticulocyte), where the labs of Schultz, Chamberlin and Hecht have studied a wide variety of proteins using these expression systems [6, 7]. The use of unnatural amino acids has since been expanded to a *Xenopus* oocyte in vivo translation system [8], which has been used by our lab to study ion channels and neuroreceptors. Although these expression systems have provided a wealth of information, they do have their limitations. Because *Xenopus* oocytes are intact cells, this translation system is more physiologically relevant than cell free systems. However, many of the proteins that are studied are of mammalian origin, and there are a growing number of examples addressing protein-protein interactions of mammalian cellular signaling pathways. Since proteins do not function as discrete entities within a cell, but rather in concert with many proteins, it would be more relevant to study these proteins in an expression system that more closely resembles their natural environment. Removing a protein from its natural environment limits any conclusions that can be drawn. This research report therefore addresses efforts to incorporate unnatural amino acids into proteins expressed in mammalian cells.

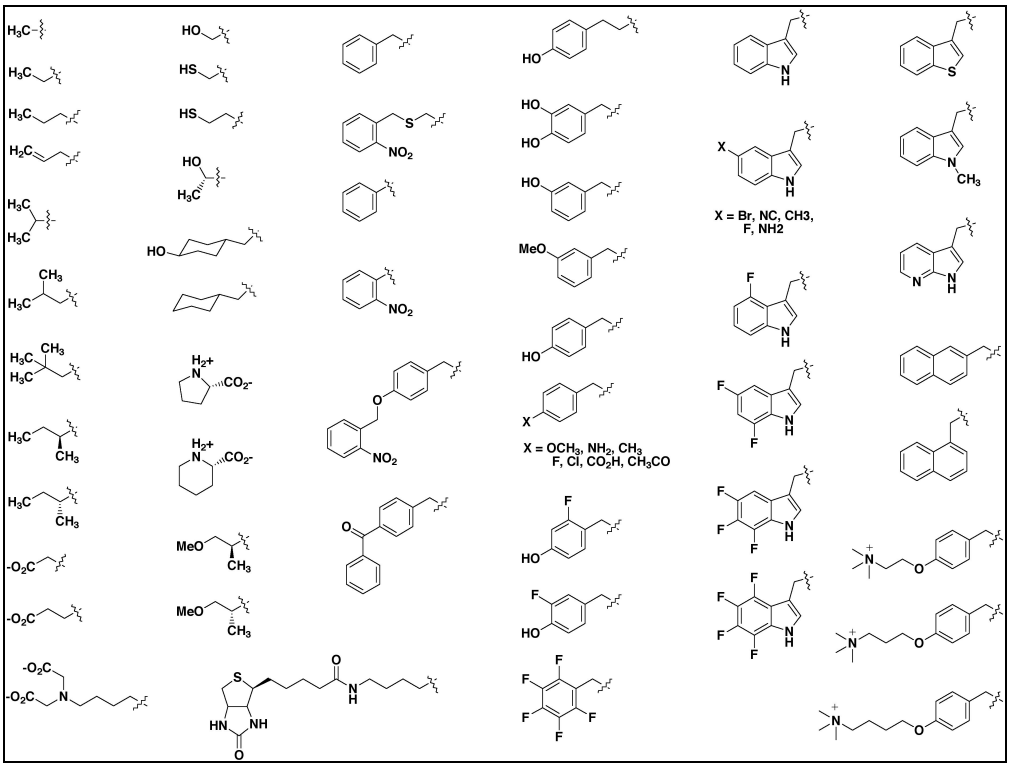

Figure 1.1. Unnatural amino acids incorporated into proteins using the *Xenopus* oocyte expression system.

Unnatural amino acid incorporation takes advantage of nonsense suppression, a phenomenon in which a stop codon can be read by a suppressor tRNA. There are naturally occurring suppressor tRNAs in a variety of biological systems, whereby the tRNA is charged with one of the twenty natural amino acids [9, 10]. In the unnatural amino acid technique, a suppressor tRNA is chemically charged with an unnatural amino acid. The first example of unnatural amino acid incorporation was reported in 1989 by Schultz [11], and shortly thereafter by Chamberlin [12]. An amber suppressor tRNA was generated from $tRNA^{Phe}$ such that it no longer recognized the normal Phe codon, but a TAG stop codon instead. By using an efficient method to misacylate tRNA developed by Hecht [13], Schultz was able to put a variety of unnatural amino acids onto yeast $tRNA^{Phe}$(CUA). This was put into an *E. coli* expression system, along with mRNA

encoding for ß-lactamase that contains a TAG codon in place of the Phe66 codon (via standard site directed mutagenesis). Because there are no endogenous tRNAs that recognize the TAG codon, only the unnatural amino acid on the amber suppressor tRNA was incorporated at that position of the protein. This was in competition with translation termination, so a mixture of truncated protein and full-length protein bearing the unnatural amino acid was generated. Importantly, while the yeast amber suppressor tRNA is recognized by the *E. coli* translational machinery, it is orthogonal to the aminoacyl synthetases. Therefore the unnatural amino acid was not replaced with Phe. Research in both the Schultz and Hecht labs has subsequently led to great success with unnatural amino acid incorporation using the yeast amber suppressor tRNA in in vitro translation systems [14-17], where as Chamberlin uses an *E. coli* $tRNA^{Gly}$(CUA) amber suppressor [3, 6, 12, 18]. Since the development of the technique, Schultz has developed an improved amber suppressor tRNA - *E. coli* $tRNA^{Asn}$(CUA) - that shows a higher ratio of full length protein to truncated in an *E. coli* expression system [19].

Sisido and coworkers have taken a different approach to unnatural amino acid incorporation into proteins. Rather than using suppressor tRNAs, they have designed tRNAs that read four-base [20, 21], or even five-base codons [22]. Multiple stop codons (just downstream of the four/five base codon) are engineered into the message if a frame-shift occurs by an endogenous tRNA encoding for the first three nucleotides. This technique then allows for the incorporation of more than one unnatural amino acid [23], since each can have a unique four/five base codon.

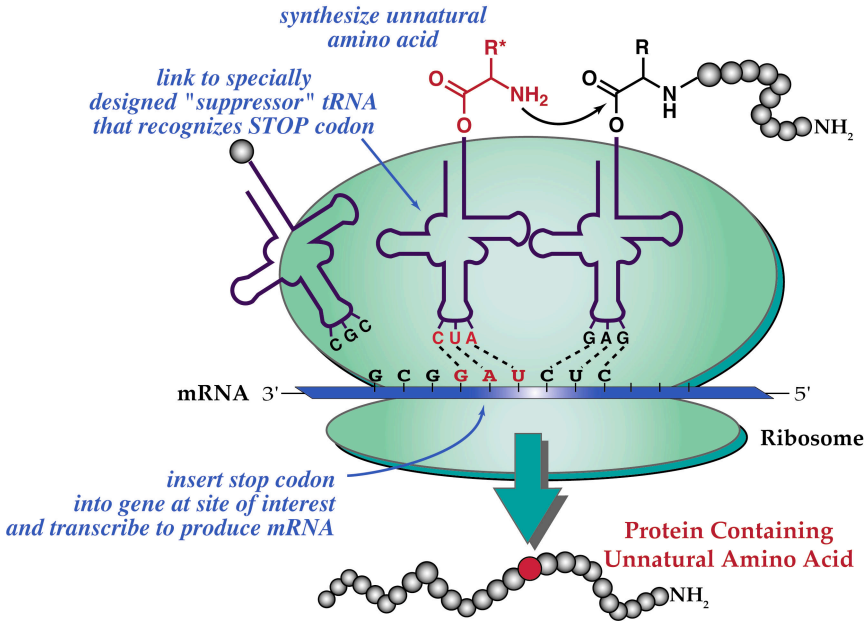

Figure 1.2. Unnatural amino acid incorporation by nonsense suppression. The unnatural amino acid is first synthesized, coupled to an in vitro transcribed suppressor tRNA, and incorporated into the desired expression system. Likewise, the mRNA encoding for the protein of interest is mutated to incorporate a TAG stop codon at the desired site of unnatural amino acid incorporation.

A limitation of the nonsense suppression methodology is that the aminoacylated tRNA is a stoichiometric reagent. To therefore generate enough protein for characterization, either the use of very large quantities of aminoacylated tRNA or a very sensitive assay is required. In our lab, the latter approach has been taken. An in vivo *Xenopus* oocyte expression system is used to study ion channels and neuroreceptors [8]. These proteins are ideal candidates for the unnatural amino acid technique. Membrane bound proteins are more difficult to study than soluble proteins in that they require cellular trafficking machinery and a membrane to fold and assemble correctly. They are also difficult to over-express, making it difficult to obtain crystal structures. The unnatural amino acid technique offers a convenient way to probe protein structure and function since they can be modified very specifically with unnatural amino acids, and

their function can be assayed by very sensitive electrophysiological techniques [4, 5]. Information on ligand binding and ion channel gating mechanisms has been obtained on a variety of ion channels using this method including the nicotinic acetylcholine receptor (nAChR) [24-29], the serotonin receptors 5-$HT3_A$ [30] and MOD-1 [31] (latter derived from *Caenorhabditis elegans*), and the Shaker [32] and Kir2.1 [33] potassium channels.

The *Xenopus* oocyte expression required the engineering of a new amber suppressor tRNA because Schultz's yeast amber suppressor was not orthogonal to this system. The first amber suppressor designed, MN3, was a modified version of the yeast $tRNA^{Phe}$(CUA) [24]. There was poor orthogonality to this expression system, however. Subsequently an improved tRNA construct has been engineered, derived from *Tetrahymena thermophila*. This organism uses a nonstandard genetic code where TAG encodes for glutamine. U to G modification at position 73 reduced recognition by endogenous *Xenopus* glutamine synthetatses, generating an amber suppressor superior to MN3 [34]. This is charged with unnatural amino acids, and is co-injected with mRNA encoding for the protein of interest into *Xenopus* oocytes.

The use of unnatural amino acids has evolved from in vitro translation systems to an in vivo translation system. The next challenge is to progress to a mammalian expression system. This would provide a more relevant model system when studying mammalian proteins.

1.2 Nonsense suppression in mammalian cells

Prior to 1982, nonsense suppression was studied in yeast and bacteria but not in mammalian cells [9, 10]. However, nonsense mutations are associated with some diseases, and it was thought that suppressor tRNAs could be used in genetic therapy. As a first effort toward this goal, RajBhandary and Sharp demonstrated that exogenous tRNAs could be properly spliced, processed and modified in transfected CV-1 (monkey kidney) cells that had been transfected with the $tRNA^{Tyr}$ gene. Their $tRNA^{Tyr}$ construct was derived from *Xenopus laevis*, and was aminoacylated by the endogenous CV-1 tyrosine synthetase (TyrRS) [35]. They subsequently mutated this tRNA to be an amber (UAG) suppressor which was still recognized by the CV-1 TyrRS. Suppression of amber nonsense mutant reporter genes by $tRNA^{Tyr}$(UAG) was achieved in an in vitro reticulocyte translation system, as well as in cultured CV-1 cells by viral cotransfection [36] or microinjection of the $tRNA^{Tyr}$ gene (DNA) [37]. The suppression efficiencies ranged from 20 to 40% of wild-type protein expression. Interestingly, in the viral co-transfection studies it was determined that 24 to 48 hours between suppressor tRNA transfection and reporter gene transfection led to optimal suppression [38].

A new generation of suppressor tRNAs was later developed by RajBhandary, Sharp and Capone, derived from a human serine tRNA [39]. All three amber, opal and ochre suppressor tRNAs were made and virally transfected into CV-1 cells. The suppression efficiency of the amber mutant was found to be 25 to 30%, and the ochre ~15%. Suppression of the opal constructs was initially unsuccessful, but was later achieved in both CV-1 and mouse NIH3T3 cells [40]. In this latter report, it was shown

that the suppression efficiencies ranged from 10 to 50% of that of wild-type protein expression, was typically higher in CV-1 cells than NIH3T3 cells and ranked in the order amber > opal > ochre.

All of the above examples are of cells that continuously express both suppressor tRNA and nonsense mutant reporter genes. To harness control of nonsense suppression, such that it can be turned on and off, inducible amber suppressor systems have been developed. One example employed the use of a temperature-sensitive viral vector carrying the gene encoding for human $tRNA^{Ser}$(UAG) in CV-1 cells [41]. At 39.5˚C nonsense suppression was blocked, but at 33˚C transcription of the suppressor tRNA was turned on. Alternatively, Capone and coworkers took advantage of the *lac* operator/repressor system in HeLa cells stably transfected with the human $tRNA^{Ser}$(UAG) gene [42]. By the incorporation of the *lac* repressor upstream of the coding region, suppression was inhibited until the inducer IPTG was applied.

An elegant example of an inducible nonsense suppression system was developed by RajBhandary, and integrates almost 20 years of research. Because the inducible expression systems discussed above depend upon aminoacylation of the suppressor tRNAs by endogenous synthetases, there is read through observed and hence a background level of protein expression. RajBhandary and coworkers took a different approach by controlling the suppressor tRNA function rather than tRNA expression [43]. They reported the development of an *E. coli* $tRNA^{Gln}$(UAG) amber suppressor that is orthogonal to the mammalian glutamine synthetase (GlnRS) of both CV-1 and COS-1 cells (monkey). Suppression was achieved when the amber suppressor tRNA was co-expressed with both a mutant CAT reporter gene and *E. coli* GlnRS, hence

aminoacylation of the suppressor tRNA was dependent upon its own synthetase. This was later fine tuned by placing the *E. coli* GlnRS gene under the control of the tetracycline regulatory element [44]. Both HeLa (human) and COS-1 cells were stably transfected with this gene, along with genes encoding for the *E. coli* $tRNA^{Gln}$(UAG) amber suppressor and the mutant CAT reporter. Suppression was blocked in the presence, and initiated in the absence of tetracycline.

1.3 Delivery of aminoacyl-tRNA to mammalian cells

To use unnatural amino acids in mammalian cells, a method must be devised for delivering charged tRNAs. This method must be capable of delivering large quantities of tRNA to the cells since aminoacyl-tRNAs are a stoichiometric reagent, and should also be able to deliver *both* aminoacyl-tRNA and the reporter gene (DNA or mRNA) to cells. Delivery should be rapid because of the susceptibility of the aminoacyl ester linkage to hydrolysis. Also, this method should not disrupt normal cell physiology, and should be applicable to a variety of cells lines. For the study of neuronal ion channels, it would be useful if the methodology could be easily transferred to neurons.

There are only a few examples of delivering exogenous tRNA to mammalian cells. Deutscher has reported aminoacyl-tRNA delivery by electroporation [45] and saponin mediated cell permeabilization [46]. In this example, total RNA from cell extracts were delivered to cells, rather than purified or in vitro transcribed aminoacyl tRNA. More recently, RajBhandary and coworkers have shown the delivery of both amber and ochre suppressor aminoacyl-tRNAs (purified from *E. coli*) to COS-1 cells

using the transfection reagent Effectene (Qiagen) [47]. They observed suppression of chloramphenicol acetyl transferase, mutated to contain the corresponding stop codon within the coding region. Lastly, Vogel and coworkers demonstrated nonsense suppression of EGFP with aminoacyl-tRNA [48]. They microinjected CHO cells with chemically aminoacylated in vitro transcribed amber suppressor tRNA. In none of these examples was an unnatural amino acid delivered to mammalian cells.

An alternate approach to site-specific unnatural amino acid incorporation was reported by Yokoyama and coworkers [49]. They expressed in CHO-Y cells a mutant *E. coli* tyrosine synthetase that aminoacylates *B. Stearothermophilus* amber suppressor tRNA with 3-iodo-L-tyrosine. This is significant work toward engineering cells with novel amino acids, but is complicated by the requirement that each new amino acid has a specific engineered synthetase and tRNA. For our purposes, chemical aminoacylation of tRNA has the distinct advantage of not being amino acid specific and no protein engineering is required, and therefore it is a more general technique.

It was our ultimate goal to develop a general method for unnatural amino acid incorporation into mammalian cells. Toward this goal, a variety of transfection methods were tested, including electroporation, lipofection, peptide-mediated delivery and biolistics. We also tested several suppressor tRNAs. By not being limited to the *Xenopus* oocyte expression system, the use of unnatural amino acids in studying protein structure-function relationships in cell-specific signaling cascades will be greatly expanded. This will advance our studies on neuronal ion channels, as well as making the use of unnatural amino acids more attainable to a broader cross-section of researchers.

1.4 References

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