

## The Incorporation of Unnatural Amino Acids into Proteins by Nonsense Suppression.

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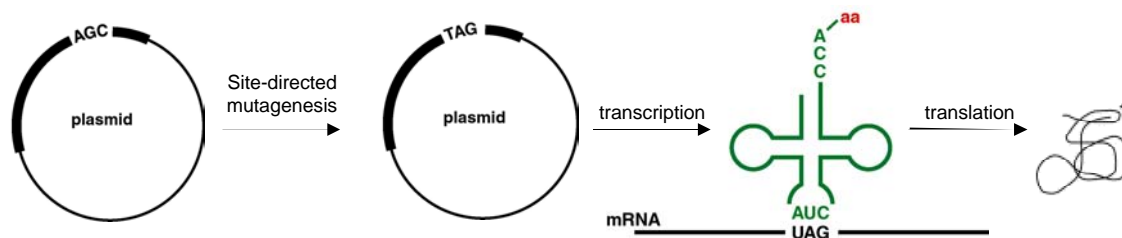
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3<sup>rd</sup> Year Seminar Requirement

A variety of methods for the incorporation of unnatural amino acids into proteins has been developed. The more synthetic methods include the total chemical synthesis of proteins and native chemical ligation of peptide fragments (1). While total synthesis offers optimal control of the individual residues incorporated into a protein, the technique is generally limited to 30-50 residues. These chemical techniques may produce products that are incorrectly folded or lack natural post-translational modifications, and they cannot be used to synthesize intact transmembrane proteins.

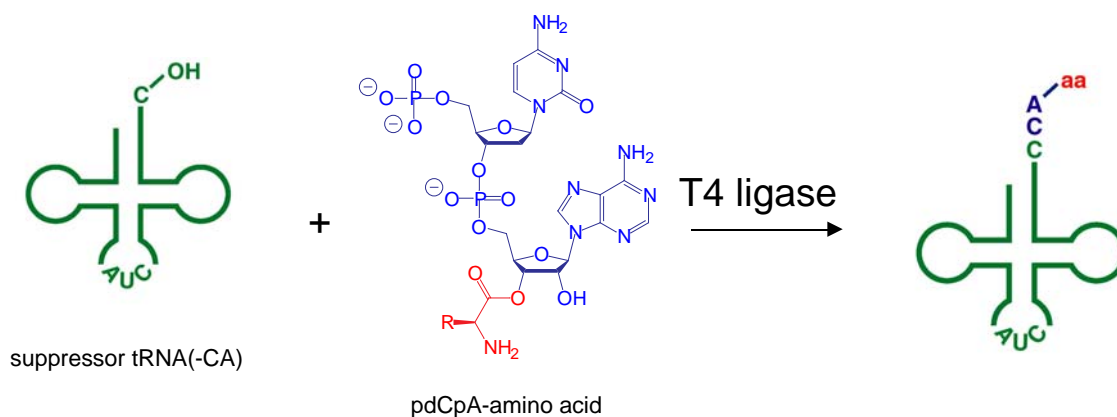
Traditional biosynthetic techniques to incorporate unnatural amino acids into proteins include cell growth in the unnatural amino acid (2-4) and modifications of side chain functionality of lysine or cysteine aminoacylated tRNAs. More recent methods involve the use of 4 base codons (5, 6) or unnatural nucleotides to create new codon/anticodon pairs (7).

In 1962, the first report of using a tRNA charged with the wrong amino acid for incorporation into protein involved the chemical desulfurization of Cys-tRNA<sup>Cys</sup> to give Ala-tRNA<sup>Cys</sup> (8, 9). Since then, Hect and coworkers have made significant contributions in developments to misacylate tRNAs with alternate amino acids (10). In 1985, Bayley and Shih announced intentions to introduce an unnatural amino acid into a protein by using an amber stop codon (11). Four years later, Schultz and coworkers published the first paper on the nonsense suppression method for the site-specific incorporation of unnatural amino acids into proteins (12). This method involves site directed mutagenesis to introduce an amber stop codon (UAG) at a specific site in a gene of interest. Translation of the corresponding mRNA proceeds with suppression of the stop codon by a suppressor tRNA acylated with the desired amino acid as shown in Figure 1.



**Figure 1:** Nonsense suppression methodology to incorporate unnatural amino acids into proteins.

The suppressor tRNA can be formed by anticodon loop replacement (13, 14), or by the more widely used runoff transcription (15, 16). PdCpA is synthesized (17) and then acylated with the amino protected cyanomethyl ester of the desired amino acid (18, 19). The pdCpA-amino acid is then ligated to the suppressor tRNA(-CA) with T4 RNA ligase shown in Figure 2 (20). The alpha amino acid (and sidechain functionality) can be protected with the photolabile 6-nitroveratryl oxycarbonyl (NVOC) group (21-23). Recent publications by Hecht and coworkers suggest a 4-pentenoyl group cleaved with iodine as another method for amine protection (24,-26). These misacylated tRNAs can then be used to incorporate the desired amino acid into proteins through *in vitro* systems using *E. coli*, rabbit reticulocyte, or wheat germ extracts or the *in vivo* *Xenopus* Oocyte system (27, 28).



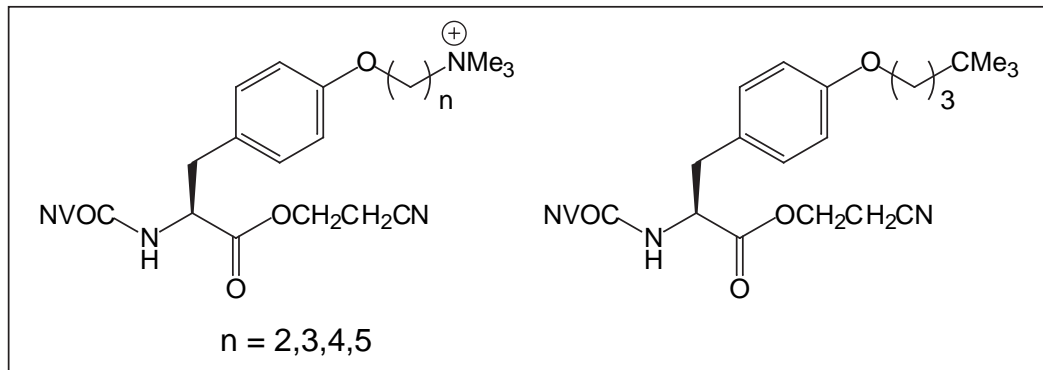
**Figure 2:** Synthesis of misacylated suppressor tRNA.

A variety of suppressor tRNAs have been misacylated and tested for their ability to incorporate amino acids into proteins in response to an amber nonsense mutation. Chamberlain and coworkers focused their efforts on *E. coli* tRNA<sup>Gly</sup> (29) due to the lack of a "double sieve" editing mechanism in the synthetase (30). Mutations in the acceptor stem of this tRNA prevented recognition by the native synthetase. Schultz and coworkers developed a suppressor tRNA<sup>Phe</sup> from yeast because it was known that this synthetase was poorly recognized by the native *E. coli* Phe synthetase (31). Unfortunately this tRNA had a low suppression efficiency, particularly with polar amino acids, which was attributed to the reduced affinity of the *E. coli* ribosome for the natural yeast tRNA<sup>Phe</sup> (32). To address these issues, suppressors derived from *E. coli* tRNA<sup>Asn</sup> and tRNA<sup>Asp</sup> were developed (33). Lester and Dougherty and coworkers developed suppressor tRNA<sup>Gln</sup> from *T. thermophila* because the tRNA introduces glutamine at a UAG codon in this natural system (32, 34). Schultz and coworkers determined that suppressor tRNA<sup>Asn</sup> from *E. coli* and tRNA<sup>Gln</sup> from *T. thermophila* showed the best overall suppression efficiency for amino acid incorporation into model proteins T4 lysozyme and chlorismate mutase (32). The authors caution, however, that suppression efficiency is subject to many variables that are not understood and may change dramatically depending on the protein and site of incorporation.

There have been many attempts to develop orthogonal suppressor tRNA / synthetase pairs for the *in vivo* amino acylation of unnatural amino acids and incorporation into proteins (35-37). Schultz and coworkers have developed a double selection screen for a library of *M. jannaschii* suppressor tRNA<sup>Tyr</sup> mutants (37). In the first selection, suppressor tRNA mutants which are not acylated by endogenous *E. coli* synthetases are chosen by the lack of translation of a toxic gene with an amber mutation. In the second round of selection, suppressor tRNA mutants which can be charged with an amino acid by the *M. jannaschii* synthetase are selected for by an amber mutation in a  $\beta$ -lactamase gene which produces a protein enabling cells to survive in the presence of ampicillin.

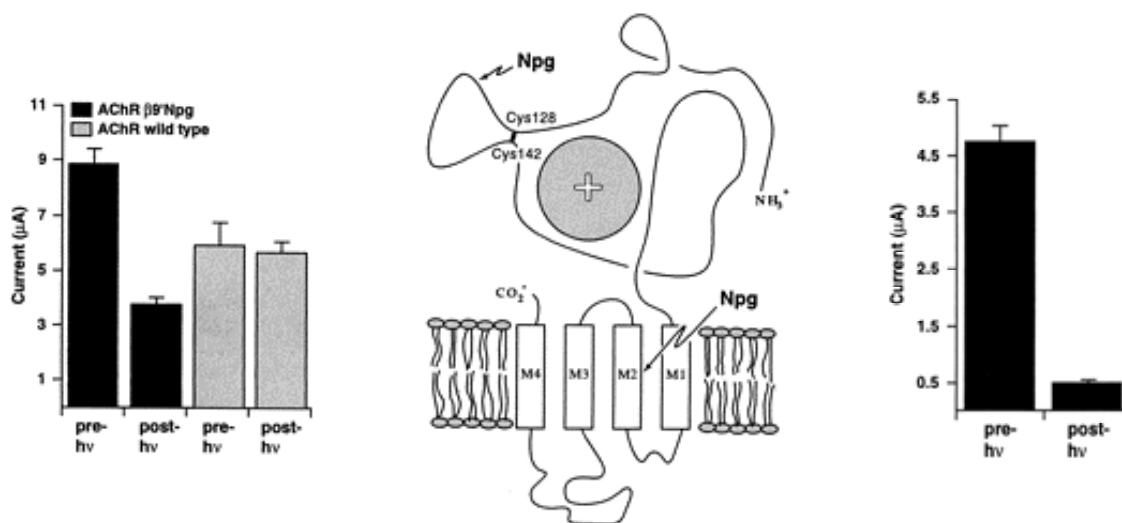
A variety of reviews and methodology publications on the use of nonsense suppression for the incorporation of unnatural amino acids into proteins are available (19, 27, 28, 31, 38-43).

Many different proteins and biological systems have been investigated by incorporation of an unnatural amino acid through nonsense suppression. Dougherty and coworkers have extensively studied activating of the nicotinic acetylcholine receptor (nAChR) by binding of the neurotransmitter acetylcholine (ACh) resulting in depolarization and subsequent neurotransmission (44-49). Incorporation of an unnatural amino acid tethered agonists shown in Figure 3 allowed studies of the binding location of ACh by varying the length of the tether (49).



**Figure 3:** Tethered agonists used in studying the nicotinic acetylcholine receptor.

Unnatural amino acids can be incorporated for selective cleavage of protein backbones. Photolabile *o*-Nitrophenyl glycine (Npg) was used to cleave an extracellular nAChR loop without the disruption of a disulfide bond to study the necessity of the loop for ACh binding and receptor depolarization (50). Allyl glycine was also incorporated into DHFR and shown cleave the protein under mild iodine conditions (51).



**Figure 4:** Probing the nicotinic acetylcholine receptor by incorporation of the unnatural amino acid *o*-nitrophenyl glycine (Npg). This amino acid cleaves the protein backbone upon light activation and resulted in deactivation of the receptor as measured by a voltage clamp. The graph on the left shows data for Npg incorporated in the extracellular loop, and the graph on the right shows data for Npg incorporated at a transmembrane position.

Numerous caged amino acids have been incorporated into proteins to study activation upon decaging (51-56). A photolabile *o*-nitrobenzyl tyrosine was used to mimic a phosphorylated tyrosine (56). In the natural system, dephosphorylation induces an endocytosis pathway. Decaging of the tyrosine resulted in the naturally observed pathway demonstrating a powerful technique for studying kinase pathways.

Spin (57) and fluorescent labels (57, 58) have also been incorporated to facilitate low concentration protein detection and to approximate distances between receptors and ligands. For example, FRET was used to examine distances between the G-protein coupled receptor Neurokinin (tachykinin)-2

and its protein ligand. A donor NBD-Dap unnatural amino acid was incorporated into two different sites of the G-protein coupled receptor and the ligand was labeled with an acceptor TMR group (59).

Nonsense Suppression can also be used to incorporate unnatural amino acids to probe protein structure-function relationships. The emission wavelength of firefly luciferase can be altered by modification of the amino acid at site 286 (60, 61). Phosphorylated and glycosylated unnatural amino acids were also incorporated, further expanding the potential applications of the methodology. A variety of proline derivatives can be used to probe the effects of ring size on protein structure and function (62).  $\beta$ -branched analogs have been used to study effects on  $\alpha$ -helical structure (63). The  $\beta$ -branch can destabilize the helix due to restricted rotation, but it can also stabilize the helix due to additional van der Waals interactions. Two sites in the T4 lysozyme that were assumed to be similar through molecular dynamics were chosen for incorporation of the unnatural amino acids. However, incorporation of t-butyl glycine at Ser44 destabilized the helix, while incorporation at Asn68 had a stabilizing effect. This result was not predicted by molecular dynamics and illustrates a powerful advantage of the nonsense suppression technique. Finally, very slight perturbations in the substrate binding pocket of the homodimer HIV-1 protease were observed with the incorporation of  $\beta$ -branched aspartic acid analogs (64). These observations would not have been possible with conventional site-directed mutagenesis since the carboxylate is necessary for protease activity.

In conclusion, nonsense suppression is a superior methodology for the incorporation of unnatural amino acids into proteins. A wide range of  $\alpha$ -amino acid analogs can be introduced at a single specific site of choice. This technique has been used to study a variety of receptors through several methods including tethered agonists, caged amino acids, and FRET. Protein backbones can be selectively cleaved by the incorporation of the photolabile o-nitrophenyl glycine and iodine labile allyl glycine. Selective incorporation of spin and fluorescent labels can aid in protein detection. Finally, protein structure and function relationships can be probed through the incorporation of proline analogs or  $\beta$ -branched amino acids.

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