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In Vivo Incorporation of Unnatural Amino Acids Using the Chemical Aminoacylation Strategy. A Broadly Applicable Mechanistic Tool

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Abstract

We describe one strategy for incorporating unnatural amino acids site-specifically into proteins expressed in living cells, involving organic synthesis to chemically aminoacylate a suppressor tRNA, protein expression in *Xenopus* oocytes, and, primarily, monitoring protein function by electrophysiology. With this protocol, a very wide range of unnatural amino acids can be employed, allowing both systematic structure-function studies and the incorporation of reactive functionality. Here we present an overview of the methodology and examples meant to illustrate the versatility and power of the method as a tool for investigating protein structure and function.

Keywords

nonsense suppression; backbone mutation; *Xenopus* oocyte; cation- π interaction; fluorescence

Background and Introduction

Chemists can immediately appreciate the potential of a method to replace any amino acid in a protein with an unnatural analogue that can be of almost unlimited structural diversity. Arguably, the structure-function study is the foundation of physical organic chemistry, and such an approach would enable for proteins the application of the mindset and the methodology that defined the key mechanistic underpinnings of modern organic chemistry. Early work established the potential for hijacking the natural protein synthesis machinery for such a purpose. A remarkable experiment by Benzer in 1962 took a cysteine tRNA with cysteine attached, converted the cysteine to alanine using Raney nickel, and then showed that alanine would be incorporated at a cysteine codon.^[1] Hecht developed a general strategy for chemically aminoacylating tRNA,^[2] and Brunner showed that a chemically aminoacylated tRNA was translationally competent.^[3] The notion of using a stop codon, in particular amber (TAG), was well-precedented by naturally occurring amber suppressor tRNAs.

In 1989 Schultz and coworkers put the whole system together.^[4] A yeast tRNA^{Phe} was modified to have an anticodon that would recognize the amber stop codon. Hecht's strategy

was used to put unnatural amino acids onto the tRNA. This aminoacyl-tRNA was added to an *E. coli in vitro* expression system, along with a plasmid coding for a protein with a stop codon inserted into the coding region. Full length protein with an unnatural amino acid incorporated was prepared. At the same time, Chamberlin and coworkers used a similar strategy to incorporate unnatural amino acids into expressed peptides.^[5]

In the following years, studies by Schultz, Hecht, Sisido and others showed that a range of unnatural amino acids could be incorporated by the strategy just described.^[6] It is fair to say, however, that the method was not widely adopted. The primary reason for this is understandable: the aminoacyl-tRNA prepared by the chemical synthesis strategy is a stoichiometric reagent in the process. At best, one protein molecule can be prepared for each aminoacyl-tRNA consumed, and in reality the yield is never 100%. Preparing the aminoacyl-tRNA is not a trivial undertaking, and so the major limitation of the chemical aminoacylation strategy is that it is difficult to prepare sufficient quantities of unnatural amino acid-containing protein for most biochemical experiments.

In the mid-1990s we were focused on molecular neurobiology, trying to understand the neuroreceptors and ion channels that control synaptic signaling. We had predicted that cation- π interactions would play an important role in neuroreceptors gated by acetylcholine (ACh),^[7] but lacked the tools to test the proposal. During this period, we came to appreciate the extraordinary extent to which electrophysiology, the primary tool of molecular neurobiology, was both extremely informative and extremely sensitive. The patch clamp, described in 1976 by Neher and Sakmann,^[8] established the ability to detect and analyze in real time single ion channel molecules. Even less sophisticated whole-cell recording methods routinely detect subfemtomol quantities of ion channels. We realized that electrophysiology could provide a strategy for circumventing the major weakness of the unnatural amino acid methodology – with a highly sensitive assay, one does not need to prepare large quantities of protein. One could argue that the integral membrane proteins of neuroscience are the ideal candidates for advanced structure-function studies, in that structural information still is far less available for these structures than for soluble proteins.

Immediately, however, there was a problem with this possible strategy. Neuroreceptors and ion channels are complex, multisubunit proteins, and heterologous expression typically requires sophisticated cellular functions related to processing and transport. As such, these proteins are generally incompatible with many common protein expression systems, including *in vitro* methods but also whole cell expression systems such as *E. coli* and yeast. Typically, a vertebrate cell is required, and a common vehicle is the *Xenopus laevis* oocyte. This is a large (~1 mm diameter) cell that is neither growing nor dividing, and it was an established tool for heterologous expression of mammalian neuroreceptors and ion channels that were then probed by electrophysiology. A very wide range of receptors, ion channels, and other signaling systems can be probed in the *Xenopus* oocyte.^[9] The key, then, was to move the *in vitro* unnatural amino acid methodology into a living vertebrate cell.

In the present review we will describe the methodology and accomplishments of the chemical acylation strategy as applied to *in vivo* protein expression, primarily in *Xenopus* oocytes. As the focus of this Special Issue is on *in vivo* expression, we will not discuss many

excellent, more recent applications of the chemical acylation approach with *in vitro* expression. As appropriate, we will compare and contrast the chemical acylation methodology with the several strategies for unnatural amino acid incorporation that employ natural or modified aminoacyl-tRNA synthetases to put the unnatural amino acid onto the tRNA.^[10] To be clear from the start, the approach described here has significant constraints compared to the synthetase strategies that limit its value for certain kinds of studies and as a tool for biotechnology. However, as a mechanistic probe, the chemical acylation strategy has some distinct advantages that make it a powerful tool for fundamental studies of the structures and functions of complex proteins. We have published some earlier reviews on the method,^[11] and an overview of some relevant results has been presented recently by Pless and Ahern.^[12]

Methodology

In collaboration with Professor Henry Lester of Caltech's biology division, we set out to develop an *in vivo* nonsense suppression methodology (Figure 1), with helpful input from many Caltech colleagues. The notion of injecting both the mRNA (with the stop codon) and the aminoacyl-tRNA directly into the *Xenopus* oocyte was straightforward, if unprecedented. The issue quickly became tRNA orthogonality, a topic discussed elsewhere in this collection. For his studies using an *E. coli*-based *in vitro* translation system,^[4] Schultz used a yeast tRNA, as it was known that yeast tRNAs are orthogonal to *E. coli*. Professor Schultz kindly provided this yeast tRNA, but it was not viable in the *Xenopus* oocyte. Using the known rules of tRNA identity,^[13] we introduced into the yeast tRNA three mutations that were meant to abolish recognition by *Xenopus* synthetases. The resulting tRNA, termed MN3, was useful in the *Xenopus* oocyte, and we reported our first results in 1995.^[14] This was the first example of incorporating an unnatural amino acid into a protein expressed in a cell of any kind, and the cell was not *E. coli* or yeast, but was a vertebrate cell. Further studies revealed that MN3 was not sufficiently orthogonal to be broadly applicable in the *Xenopus* oocyte system.

To create an improved suppressor tRNA, we exploited the fact that *Tetrahymena thermophila* has a nonstandard genetic code, such that the amber stop codon (TAG) is, in fact, a coding codon for Gln. We speculated that the corresponding tRNA in *Tetrahymena* might be an especially efficient suppressor of an amber stop codon. We mutated position 73 to remove recognition by the Gln synthetase, producing THG73, a highly efficient, "highly orthogonal" tRNA.^[15] THG73 has proven to be very effective at nonsense suppression in the *Xenopus* oocyte and in other expression systems, and the bulk of our experiments have been performed with it.

We have developed other tRNAs with improved properties that are viable in the *Xenopus* oocyte, including tRNAs that recognize a second stop codon (opal) and two frameshift suppressors,^[6c] which recognize 4-base codons.^[16] Rather than a randomization/selection process to develop new tRNAs, we exploited the known rules of tRNA identity to rationally select sites of modification and to optimize tRNA suppression efficiency and orthogonality. These new tRNAs allow the incorporation of multiple unnatural amino acids in a single experiment. We have frequently probed a protein with two unnatural amino acids, and we

have shown that three unnatural amino acids can be site-specifically incorporated into a protein simultaneously.

In other regards the methodology employed for studies in *Xenopus* oocytes follows established procedures.^[17] In the overwhelming majority of cases, the aminoacyl-tRNA was prepared by protocols developed by Hecht and Schultz, involving chemically coupling the unnatural amino acid first to the dinucleotide dCA, and then using a ligase to link the aminoacyl-dCA to the remainder of the tRNA (which is prepared by runoff transcription).^[18] An important advance was the development of a MALDI-TOF mass spectrometry protocol that allows us to evaluate the extent to which the protocol has been successful in preparing a full length tRNA with the unnatural amino acid appended.^[19] Other strategies have included the direct chemical acylation of full length tRNA, either by employing cationic micelles^[20] or catalysis by lanthanide elements.^[21] tRNA acylating ribozymes termed flexizymes have also been developed.^[22]

Microinjection of mRNA and aminoacyl-tRNA into the *Xenopus* oocyte is straightforward and is followed by incubation for 24 – 48 hours to allow for protein expression, folding, assembly, and transport to the cell surface. In challenging cases, a second injection of aminoacyl-tRNA (with or without mRNA) is performed 24 hours after the first. Typically, the total amount of RNAs injected is on the order of 10s of ng, and so only a small quantity of aminoacyl-tRNA (~ 1 pmol) is consumed in a given experiment. The method, overall, is not efficient – the number of functional channels available for probing on the surface of the oocyte could be 1000-fold fewer than the number of aminoacyl-tRNA molecules injected. Part of this is because nonsense suppression is not completely efficient. Another factor that contributes to the inefficiency, however, is the fact that for complex, multisubunit membrane proteins, translation of a protein chain does not always lead to functional protein on the surface of the oocyte, even for the wild type. A substantial fraction of protein is trapped in the endoplasmic reticulum (or other parts of the transport apparatus) due to misfolding, misassembly, or failure of transport to the surface. Again, because of the high sensitivity of electrophysiology, this inefficiency is not a concern.

The most common electrophysiology protocol is the two-electrode voltage clamp experiment, in which all the receptors/channels on the surface of the cell are interrogated. In such an experiment, it is straightforward to detect < 10 attomol of functional protein on the oocyte surface. When desired, the single-molecule methodology of the patch clamp can be employed to produce deep insights into receptor function. We note that proteins other than ion channels can be studied by this methodology. For example, activation of some G protein-coupled receptors (GPCRs) results in opening of an ion channel (GIRK), and we showed^[23] that ionic currents through the GIRK channel provide a reliable readout of GPCR activity. Also, sensitive fluorescent methodologies are applicable to the *Xenopus* oocyte system, especially when combined with TIRF microscopy (see below).

Important controls include injecting mRNA only (to test for readthrough of the stop codon) and injecting mRNA plus tRNA that does not have an amino acid appended. In each case, little or no current must be observed. Also, at each site, the first experiment is always to “rescue” the wild type, by appending the appropriate naturally occurring amino acid to the

tRNA. The full functionality of the wild type receptor must be observed in this experiment. Another positive control is to use nonsense suppression to make a known conventional mutant that has a distinct phenotype from the wild type. Again, that new phenotype must be fully recapitulated.

Scope and Limitations

Since organic synthesis is used to attach the unnatural amino acid to the tRNA, almost any unnatural aminoacyl-tRNA can be prepared, removing a limitation associated with the synthetase strategies. The only broad limitation on the method is the tolerance of the ribosome for unnatural amino acids, a limitation shared with the synthetase strategies. As shown in Scheme 1, the ribosome is remarkably tolerant. Very large side chains can be incorporated (Scheme 1C, E, H), as can α -hydroxy acids (Scheme 1D), fluorescent groups and photoreactive groups (Scheme 1E), and tethered agonists (Scheme 1H). The number of unnaturals in Scheme 1 is large, but more significant is the broad structural diversity that is compatible with the strategy. To date, the synthetase strategies simply have not been viable for such a broad and diverse range of structures.

For all this acceptance, the ribosome is essentially intolerant of D amino acids, which have the unnatural stereochemistry at the α carbon. This facial selectivity is understandable based on structures of aminoacyl-tRNAs at the ribosome. While disappointing in some ways, this stereoselectivity can be put to good use. In the chemical acylation step used to prepare the aminoacyl-tRNA, a racemic unnatural amino acid can be used, and the resulting mixture of diastereomeric aminoacyl-tRNAs can be injected into the oocyte. The ribosome will then perform a kinetic resolution, leading to incorporation of only the proper stereoisomer.

One other aspect of the chemical acylation strategy is worth highlighting. The method can incorporate unnatural amino acids that differ from a natural amino acid in the most subtle of ways (Scheme 1). This, in principle, can be a challenge for the synthetase-based approaches, as it seems likely that the natural synthetases would be competent to incorporate an unnatural amino acid that is very closely related to its cognate natural amino acid. Indeed, this is the foundation of the auxotroph strategy for unnatural amino acid incorporation.^[24] Szostak has identified over 90 unnatural amino acids that are recognized by natural synthetases and become loaded onto tRNAs.^[25] In addition, over 50 unnatural amino acids of this sort have been shown to be translationally competent at the ribosome.^[26] Again, for some applications this may not be a concern, but the use of subtle variations is a crucial component of mechanistic investigations, and some caution is in order with the synthetase strategies.

We note that in principle the strategy outlined here can be applied to other cell types, such as cultured mammalian cells. RajBhandary has established that amber suppressor tRNAs can be imported into mammalian cells, where they are then charged by endogenous synthetases and incorporate the corresponding natural amino acid at a stop codon.^[27] We have shown that aminoacyl-tRNA can be delivered into CHO and HEK cells using microelectroporation, and then unnatural amino acids can be incorporated into the nicotinic acetylcholine receptor (nAChR).^[28] While this study established the plausibility of using the chemical acylation

strategy in mammalian cells, further optimization is required before the method could see general use.

Overall, then, the scope of the chemical acylation strategy for unnatural amino acid incorporation *in vivo* is broad indeed (Scheme 1). In our labs we have incorporated over 100 unnatural amino acids in almost 200 unique sites in over 20 proteins, resulting in well over 650 unique combinations. Proteins studied include neuroreceptors activated by acetylcholine, serotonin, GABA, and glycine, several GPCRs, K⁺ channels, Na⁺ channels, NMDA receptors, and others.

The majority of our work has been done on Cys-loop receptors, so a brief introduction is in order.^[29] These are large (MW ~300,000), integral membrane proteins typically found at a synapse. They are neurotransmitter-gated ion channels – binding of a neurotransmitter such as acetylcholine (ACh), serotonin, GABA, or glycine induces a structural change that opens an ion channel contained within the receptor. Thus, these receptors mediate fast synaptic transmission throughout the central and peripheral nervous systems. They are pentameric receptors, and the five subunits can be identical or close homologues. The neurotransmitter/agonist binding site lies in the extracellular domain of the receptor, at the interface between two subunits. The receptor we have studied most is the family of nicotinic ACh receptors (nAChR), so named because nicotine binds to and activates these receptors, leading to addiction and the many other effects associated with nicotine.

We can now summarize a range of studies involving unnatural amino acid incorporation into proteins expressed in *Xenopus* oocytes. Our goal is to illustrate the kinds of problems that can be addressed with this approach. We have found it convenient to divide such studies into two categories. The first exploits the capability of the unnatural amino acid methodology for subtle, systematic changes to the protein, enabling classical structure-function studies of complex proteins. The second exploits the ability to incorporate unnatural amino acids that are very different from the natural set, often incorporating new functionality into the receptor. We divide our discussion below along these lines.

Subtle Mutations to Probe Noncovalent Interactions

Cation- π Interactions

As noted above, our personal motivation for developing the *in vivo* unnatural amino acid methodology was to determine whether cation- π interactions play a role in ligand recognition at ACh receptors, although we appreciated that a general methodology would be broadly applicable. Given the primarily electrostatic origin of the cation- π interaction,^[30] a strategy to evaluate cation- π interactions was evident. Fluorine is strongly electron-withdrawing on an aromatic ring, and thus deactivating in the cation- π interaction. Conveniently, the effect is additive, so two fluorines are twice as deactivating as one, three even more so, and so on. The strategy for detecting a cation- π interaction, then, is progressive fluorination of the Phe, Tyr, or Trp of interest. We have employed other strategies too, such as comparing cyano and bromo substitution, which present comparable sterics but the cyano group is much more strongly deactivating (Scheme 1F, G).

Remarkably, in many cases a linear free energy relationship is seen between the cation- π binding ability of the aromatic and the activation of a receptor by ACh or another cationic ligand. Our first “fluorination plot” established a cation- π interaction between ACh and a specific Trp of the nicotinic ACh receptor (nAChR).^[31] Since that time, over 30 linear fluorination plots have been seen, involving over a dozen different proteins, and an array of ligands including agonists, antagonists, toxins, Ca^{2+} ions, and more.^[11c] A highlight is the discovery that the cation- π interaction plays a central role in the pharmacology of nicotine.^[32] nAChRs are found in both the central and peripheral nervous systems, but nicotine is potent in the CNS but not in the periphery. Despite very high sequence identity at the agonist binding site, the discriminating factor is a cation- π interaction that is strong in CNS receptors but weak in peripheral receptors.

Voltage-gated ion channels (VGIC) represent another class of membrane proteins that has been quite compatible with the unnatural amino acid methodology. Tetraethylammonium (TEA) is an ion channel blocker that has a long history of use in fundamental studies of VGICs. In the *Shaker* K^+ channel a fluorination study established a key role for a cation- π interaction to a tyrosine residue in TEA blockade,^[33] and further study revealed a novel allosteric interaction between a remote residue and the cation- π interaction.^[34] In the skeletal isoform of voltage-gated Na^+ channels ($\text{Na}_V 1.4$), a cation- π interaction has been established between a highly conserved Phe and the anesthetic lidocaine.^[35] In the cardiac isoform ($\text{Na}_V 1.5$), a strong cation- π interaction has been established to contribute to the binding of class Ib antiarrhythmic drugs.^[36] Also, both Ca^{2+} ^[37] and tetrodotoxin^[38] have been shown to make cation- π interactions when acting as blockers of voltage-gated Na^+ channels.

In a sense, these fluorination studies provide high precision *structural* information – establishing van der Waals contact between the ligand and a specific side chain of the protein – but the structural insight is provided not by crystallography, but by chemistry. In favorable cases an unnatural amino acid mutagenesis experiment can provide quantitative information. We have shown that adding four fluorines to a Trp (or three to a Phe) completely removes the negative electrostatic potential on the face of the aromatic that produces the cation- π interaction, without “overshooting” (becoming repulsive to a cation). Across a family of related receptors we have seen drug affinities drop by factors of 100 to 10,000 from fluorination, the latter corresponding to a $-G^\circ$ value of 5.5 kcal/mol for a cation- π interaction.^[39]

The fluorination plots also highlight an important advantage of the unnatural amino acid methodology. As with any structure-function study, the structure of the target molecule is changed in the process. When a concomitant change in function is seen, many interpretations are possible. We replace a Trp with a fluorinated Trp, and the receptor changes. How do we know it is a cation- π interaction and not a steric or other electronic effect? The ability to incorporate monofluoro-, difluoro-, trifluoro-, and tetrafluoro-Trp, and then seeing a compelling correlation to a specific property removes the ambiguity.

We note here another unnatural amino acid that has been quite useful in evaluating aromatic amino acids. Cyclohexylalanine (Cha, Scheme 1A) is surprisingly similar to Phe in size,

shape, and hydrophobicity (place CPK models of benzene and cyclohexane side-by-side). If Cha can function well at a Phe site, cation- π interactions and other electrostatic interactions are clearly not significant. Importantly, we have characterized sites where Cha can replace Phe, but natural hydrophobic residues such as Leu cannot,^[40] indicating that not just hydrophobicity but also size and shape are important at that site.

Hydrogen Bonds to Side Chains and the Protein Backbone

Hydrogen bonds are, of course, broadly employed in binding small molecules to proteins, and unnatural amino acid mutagenesis provides powerful tools to evaluate potential hydrogen bonds. For hydrogen bonds to amino acid side chains, the approach provides more refined strategies than conventional mutagenesis. For example, the OH of Tyr is a potential hydrogen bond donor and acceptor; mutation to Phe removes both options and leaves a hole in the protein. The unnatural amino acid 4-MeO-Phe removes the hydrogen bond donor but retains the hydrogen bond acceptor. Other substituted phenylalanines such as 4-Me-Phe or 4-Cl-Phe remove all hydrogen bonding without leaving a steric hole. Another subtle mutation is to replace a carboxylate with a nitro group, as in Glu to nitrohomoalanine (Nha, Scheme 1A). The two functional groups are isoelectronic and isostructural, and while both can act as hydrogen bond acceptors, Nha lacks the negative charge of Glu that could be involved in an ion pair. We have uncovered instances where a Glu has been proposed to provide a key negative charge for binding a cationic drug, but Nha functions well, ruling out that possibility.^[41]

Hydrogen bonds to the protein backbone are also common and not amenable to evaluation by conventional approaches. However, formation of a backbone ester by incorporation of an α -hydroxy acid provides a powerful strategy. Importantly, α -hydroxy acid incorporation allows perturbation of both the hydrogen bond donating and accepting abilities of the protein backbone. A backbone ester of course removes the NH that could serve as a hydrogen bond donor. In addition, the ester carbonyl is a much poorer hydrogen bond acceptor than an amide carbonyl. Interestingly, studies in both α -helices and β -sheets have established that the magnitudes of the two perturbations are similar,^[42] and we have seen large impacts for both in drug-receptor interactions.^[43] There are some challenges associated with incorporating α -hydroxy acids using evolved synthetases,^[44] but α -hydroxy acids are especially well suited to the chemical acylation strategy (Scheme 1D). We have executed over 125 different α -hydroxy acid incorporations across a range of receptors, mutation sites, and α -hydroxy analogues.

An example of the use of α -hydroxy acids to probe hydrogen bonding is the binding of nicotine to the nicotinic acetylcholine receptor.^[32, 45] As shown in Scheme 2, unnatural amino acids have been used to define a three-point binding model for nicotine. Fluorination established a cation- π interaction. Backbone ester substitution was used to show that the N^+ -H of nicotine hydrogen bonds to the backbone carbonyl of the Trp that makes the cation- π interaction. In addition, backbone substitution on the adjacent subunit established that the pyridine N of nicotine hydrogen bonds to a backbone NH. We have established many similar hydrogen bonds between a drug and its receptor using this strategy.

Jan, Schultz and coworkers studied the selectivity filter of K⁺ channels with α -hydroxy acids.^[46] Crystallographic studies established that backbone carbonyls play a critical role in regulating ion flow through K⁺ channels, but interestingly, ion selectivity was retained even with backbone esters. Localized conformational changes were evident, resulting in altered gating behaviors.

We have also evaluated protein-protein hydrogen bonds, probing, for example, a conserved β -sheet region of nicotinic receptors that is likely involved in channel gating.^[47] An interesting example comes from the multisubunit nicotinic receptor, where we were able to identify a hydrogen bond between a protein backbone NH of one subunit and the side chain carboxylate of an Asp on a different subunit.^[48] This intersubunit hydrogen bond likely plays a key role in receptor gating.

The α -hydroxy strategy also provides a useful way to evaluate the efficiency of unnatural incorporation. Esters are more vulnerable to hydrolysis than amides, although harsh conditions are necessary, and we have seen no indication of backbone hydrolysis in the cell. Under strong *in vitro* conditions, however, proteins containing a backbone ester can be cleaved while wild type cannot.^[49] In studies involving monitoring hydrolysis by Western blotting, we see complete cleavage, indicating that α -hydroxy acid incorporation is highly efficient. The hydrolysis reaction can also be used to evaluate disulfide patterns.^[50] If the ester is incorporated within a disulfide loop, hydrolysis will not reveal a cleaved protein in a non-reducing gel, but will in a reducing gel.

Evaluating Prolines

Proline is unique among the natural amino acids in having a cyclic structure and no backbone NH when incorporated into a protein. This structural change has two important consequences for protein structure/function. Proline residues are disruptive in α -helices and β -sheets. In addition, proline residues have a much higher propensity for an *s-cis* conformation of the peptide bond (~5%) than all other amino acids (<1%). Since no other natural amino acid shares proline's unique structural features, it is difficult to evaluate the role a proline is playing with conventional mutagenesis. We have developed two strategies to evaluate proline function.

The first approach uses α -hydroxy acids (Scheme 1D). When incorporated into a protein, no backbone NH is present, as is also seen with proline. If that is the critical feature by which proline affects protein structure/function, then an α -hydroxy acid could substitute for a proline. We have seen just such a result in several instances. For example, there is a conserved proline in the middle of a transmembrane helix of Cys-loop receptors. Replacing this proline with any other natural amino acid produces a nonfunctional receptor – a minimally informative result. We found, however, that incorporating an α -hydroxy acid at the proline site gave receptors with wild type function.^[49, 51] Interestingly, the identity of the α -hydroxy acid was not important; that is, the side chain had no impact on function. All that mattered was that an α -hydroxy acid was used. This established that it was the lack of a backbone NH at the proline that was the key feature influencing receptor function.

We found a completely different result at a proline in a critical loop of the 5HT₃ serotonin receptor.^[52] At this site, α -hydroxy acids gave nonfunctional receptors. The nonsense suppression methodology, however, can incorporate a large number of proline analogues (Scheme 1B). At the proline site many, but not all, close proline analogues – that is, other cyclic amino acids – gave functional receptors. Remarkably, across a series of proline analogues we found that receptor function correlated with the inherent *s-cis* preference of the proline. For example, 5,5-dimethylproline is naturally 71% *cis*, and a receptor containing this unnatural amino acid is more active than wild type by over a factor of 60. The results established an important role for proline *cis-trans* isomerization in receptor activation. Note that esters actually show a stronger preference for *s-trans* than amides, and thus α -hydroxy substitution gave nonfunctional receptors.

It is well known that the *cis* content around a prolyl peptide bond is context dependent. Particularly influential is an aromatic immediately preceding the Pro, which significantly increases the *cis* content. We probed a conserved Phe-Pro motif in the nicotinic receptor and showed that in this instance, there was a very strong coupling between the two residues, indicating a strong, specific interaction.^[53] Note that both sites were probed with unnatural amino acids, including studies where consecutive amino acids were replaced by different unnatural amino acids.

We thus have two ways to probe the role of a proline – α -hydroxy acid substitution to see if the lack of an NH is crucial and *cis*-biased prolines to look for *cis-trans* isomerization. As another example, we probed several prolines in the D2 dopamine receptor.^[54] Prolines figure prominently in GPCR transmembrane regions, even to the point that the most conserved residues in helices 5, 6, and 7 (the X.50 residue) are prolines. In probing five different prolines we found that α -hydroxy acids were generally well tolerated, and *cis-trans* isomerization did not appear to be important. An alternative probe of the role of the backbone NH – using N-methyl substituted amino acids (Scheme 1B) – revealed differences among the prolines, such that steric bulk analogous to the ring of a proline is necessary at some, but not all, sites.

Hydrophobicity and Stereochemistry

The subtle variations enabled by the chemical acylation strategy allow intriguing structural probes. Where side chain hydrophobicity is considered essential, classical methyl, ethyl, propyl, etc. experiments can be performed (Scheme 1A). Perhaps more compelling are comparisons between isosteric residues that have differing hydrophobicities. For example, isoleucine and O-methyl threonine are isosteric, but the latter has a polar oxygen atom. At sites where hydrophobicity is especially important, we have seen shifts in receptor function as large as 6-fold for such comparisons.^[55]

Side chains like Ile and Thr allow another classical structure-function probe – stereochemistry. It is a simple matter to incorporate allo-Ile or allo-Thr, creating receptors that are diastereomers of the wild type protein.^[55] We used this approach to test a proposal based on a low resolution structure that a Val side chain was inserted into a well defined, hydrophobic pocket across a key interface of the nAChR.^[56] In the model, one methyl of the Val was buried deep into the pocket, the other much less so. We found that the Val-to-Thr

mutant (replacing the buried methyl with OH) produced nonfunctional receptors (>200-fold loss of function). While this supported the structural model, certainly many other explanations were possible. Crucial, then, was the observation that allo-Thr – replacing the other methyl with OH – produced fully functional receptors that were shifted only 2-fold from wild type. A stereochemical probe provided a compelling test of mechanism.

More Dramatic Mutations to Deliver Functionality

As noted above, the alternative use of unnatural amino acid mutagenesis is to deliver amino acids that are wildly different from the natural set, often including reactive or spectroscopically active side chains. Again, the chemical acylation strategy has some advantages in this regard, in that a synthetase for these “very unnatural” amino acids need not be developed. We summarize here several uses of the more complex amino acids.

Biocytin

The biotin group is a workhorse of biochemistry and chemical biology, and one can envision many uses of a site-specifically incorporated biotin. Biocytin is a naturally occurring (but not translationally incorporated) amino acid (Scheme 1C), and we found that it is compatible with our nonsense suppression approach.^[57] A simple use of biocytin is to evaluate surface accessibility of residues in integral membrane proteins. We placed biocytin at several positions along a region of the proposed surface of the nAChR, and then evaluated whether ¹²⁵I streptavidin could bind to the cell. In this way, we could see which side chains were exposed to the external medium.

Biocytin is one of the “most unnatural” amino acids we have incorporated. Interestingly, some locations that would have been expected to be surface exposed did not lead to streptavidin binding. We therefore tried a longer chain analogue of biocytin (Scheme 1C). This very large unnatural amino acid did incorporate into receptors, but the yield of protein was low – near our detection limit. Thus, a very large residue like this might define the limits of unnatural amino acid incorporation.

Photoresponsive Unnaturals

Photochemical probes of protein structure have long been used in many contexts, and the unnatural amino acid methodology opens up many possibilities. Both fluorescent and photoreactive residues have been used in many contexts (Scheme 1E). Here we emphasize work in *Xenopus* oocytes.

The fluorescent unnatural amino acid NBD-Dap (Scheme 1E) was one of the first unnatural amino acids incorporated into a receptor expressed in *Xenopus* oocytes.^[58] It was placed in the NK2 receptor, oocyte membranes were harvested, and FRET between the NBD and a fluorescently-labeled peptide antagonist was observed. More recently, Cohen et al. incorporated the environmentally sensitive fluorophore aladan (Scheme 1E) into proteins expressed in *Xenopus* oocytes, allowing a sensitive probe of protein microenvironment.^[59]

Single molecule imaging techniques have shown great potential for revealing many features of protein function. In the *Xenopus* oocyte, TIRF microscopy has the special advantage of

probing primarily the surface of the cell, thus avoiding the background fluorescence associated with the yolk in the cell body. We showed that BODIPY-Lys (Scheme 1E) can be incorporated into receptors expressed in *Xenopus* oocytes.^[60] TIRF imaging of intact oocytes was possible, and various strategies revealed that single molecules were being imaged by way of an unnatural amino acid-based fluorophore. These are still challenging experiments, but the potential is considerable. Especially exciting is the fact noted above that we can incorporate two different unnatural amino acids into the same protein, creating the possibility of FRET experiments in which both fluorophores are small, amino acid side chains rather than the much larger fluorescent proteins often used. Such an experiment has been accomplished by Hecht and Benkovic using chemical acylation and *in vitro* expression.^[61]

Photocleavable protecting groups – producing “caged” compounds – have seen extensive use, and it is a clear opportunity for unnatural amino acid mutagenesis.^[62] We showed very early on that a Tyr with an o-nitrobenzyl group protecting the OH – Tyr-ONB (Scheme 1E) – would incorporate efficiently into receptors.^[63] If we replaced a Tyr at the agonist binding site of the nAChR with Tyr-ONB, the receptor was unresponsive to applied ACh. However, a msec flash of light decaged the Tyr-ONB, and we could watch in real time as the (now wild type) receptor came to life, monitoring with electrophysiology. Interestingly, different binding site tyrosines showed different recovery kinetics, ranging from 10 to 800 msec. This suggested that different degrees of conformational disruption of the agonist binding site were introduced by the Tyr-ONB residue.

We next showed that Tyr-ONB could be used to regulate kinase activity.^[64] In a particular K⁺ channel, a specific Tyr was thought to be phosphorylated, but direct evidence was lacking. We incorporated Tyr-ONB at the site, and saw that the channel functioned normally. We also expressed the kinase Src and added the phosphatase inhibitor PAO. Photolysis to reveal the Tyr OH was followed by a substantial drop in channel current over a time course of minutes. This change only occurred if both Src and PAO were included. The implication was that decaging initiated a series of events that lead to removal of the channel from the oocyte surface. More generally, this study showed that kinases could be regulated by the caging strategy. In other studies we showed that we could also incorporate and deprotect caged Cys residues.^[65]

An interesting variant of the nitrobenzyl decaging strategy is the unnatural amino acid 2-nitrophenylglycine (Npg, Scheme 1E).^[66] Photochemical “decaging” leads to a cleavage of the protein backbone, a form of photochemical proteolysis. This residue has been incorporated into K⁺ channels, nAChRs,^[66] and the GABA_A receptor, where it was used to probe the linkage between the orthosteric (GABA) binding site and the allosteric binding region that is the site of action of benzodiazepines.^[67] A second-generation photochemical proteolysis residue based on decaging a selenide that then undergoes an intramolecular S_N2 reaction has also been developed.^[68]

Tethered Agonists

Unnatural amino acids that have side chains that mimic/incorporate the natural agonist for a receptor can provide valuable information. We have incorporated several unnatural amino

acids that contain an analogue of acetylcholine (ACh) throughout the ACh receptor (Scheme 1H).^[31, 69] In certain cases we see constitutively active receptors – currents are seen without having to add any agonist. By varying the position of incorporation and the length of the tether, we could learn about the relative proximity of residues to the agonist binding site. An important finding was that we could see constitutive activity when we incorporated the tethered agonist in a receptor subunit that was adjacent to the hypothesized primary agonist binding site. This provided compelling evidence that the agonist binding site lies at the interface of two subunits long before structural information was available.

Most of the tethered agonists we used incorporated a quaternary ammonium compound to mimic the quat of ACh. However, we also incorporated a series of primary, secondary, and tertiary amines.^[70] We anticipated that these would be tethered agonists only if the amine is protonated at the agonist binding site. Indeed, with tethered amines we found that the receptor was now responsive to pH. Remarkably, the *Xenopus* oocyte can tolerate pH values from 5.5 to 9.0. We could establish that the effective pK_a of a tertiary amine at the agonist binding site was 6, compared to a pK_a of 9.3 in free solution. This suggests that the agonist binding site is hydrophobic, as expected given the large number of aromatic rings that form the binding site.

Conclusions

The chemical approach to the incorporation of unnatural amino acids into proteins has proven to be highly compatible with the *Xenopus* oocyte heterologous expression system. The method can accommodate a structurally diverse array of unnatural amino acids, and it has been applied to scores of proteins. The *Xenopus* oocyte is amenable to expression of a wide range of proteins, and any system that has a fairly sensitive readout could be adapted to the unnatural amino acid methodology.

To date, the chemical acylation strategy as implemented in *Xenopus* oocytes has been primarily employed as a mechanistic tool. Structure-function studies with precision far beyond that which is possible using conventional mutagenesis have provided valuable insights across a range of proteins. One can imagine many other systems that could be adapted to this methodology, and it is hoped that other laboratories will make use of this powerful approach to understanding protein structure and function.

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Biography

Ethan Van Arnam received his bachelors degree in chemistry from Bowdoin College. He earned his PhD from Caltech in 2013, working in Dennis Dougherty's lab. There he studied ligand binding and conformational changes in ligand-gated ion channels and G protein-coupled receptors using unnatural amino acid mutagenesis. He is currently a postdoctoral scholar at Harvard Medical School.

Dennis A. Dougherty is the Hoag Professor of Chemistry at Caltech. He earned a B.S. from Bucknell University, a Ph.D. from Princeton with Kurt Mislow and did postdoctoral study with Jerome Berson at Yale. He is a member of the National Academy of Sciences and a fellow of the American Academy of Arts and Science and the American Association for the Advancement of Science. Honors include the Biopolymers Murray Goodman Award, the ACS James Flack Norris Award for Physical Organic Chemistry, and recognition as a Javits Neuroscience Investigator by NIH. He is also the co-author, with Professor Eric Anslyn, of the influential textbook, *Modern Physical Organic Chemistry*.

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Figure 1.
Schematic of methodology for incorporating unnatural amino acids into proteins expressed in *Xenopus* oocytes.

Scheme 1.

Residues that have been incorporated by nonsense suppression into functional proteins expressed in *Xenopus* oocytes. Side chains only are shown, except for panels B and D. A. Analogues of aliphatic amino acids. B. Proline analogues and N-methyl amino acids. C. Biotin derivatives. D. α -Hydroxy acids. E. Photoreponsive amino acids. F. Analogues of Phe, Tyr, and His. G. Analogues of Trp. H. Tethered agonists. In addition, most of the natural amino acids have been incorporated by nonsense suppression, but they are not shown here.

Scheme 2.
Binding model for nicotine at the nAChR.