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Unnatural Amino Acid Mutagenesis: A Precise Tool for Probing Protein Structure and Function†

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ABSTRACT: The first general method for the biosynthetic incorporation of unnatural amino acids into proteins was reported in 1989. The ensuing years have seen the solid development and subsequent implementation of "unnatural amino acid mutagenesis" in a number of groundbreaking studies. Over 100 different amino acids have been incorporated into dozens of soluble and transmembrane proteins, using both cell-extract and cell-intact translation systems. The approach has provided insights into ligand-binding sites, conformational changes, and protein−protein interactions with a level of precision simply unparalleled by conventional mutagenesis. Here, the methodology is outlined, significant applications of the approach are summarized, and recent major improvements in the method are discussed. The future will likely see many more investigators utilizing this approach to manipulate proteins as it realizes its promise of becoming a tool with enormous potential.

In the 1980s, three laboratories began taking the first steps toward expanding the genetic code to incorporate unnatural amino acids into proteins with Schultz and co-workers reporting the first general method for the biosynthetic incorporation of unnatural amino acids into proteins in 1989 (*1*−*3*). The ensuing years have seen the solid development and subsequent implementation of "unnatural amino acid mutagenesis" in a number of groundbreaking studies. Over 100 different amino acids have been incorporated into dozens of soluble and transmembrane proteins, using both cell-extract and cell-intact translation systems. The method has provided insights into ligand-binding sites, conformational changes, and protein−protein interactions with a level of precision simply unparalleled by conventional mutagenesis. A number of reviews highlighting applications of the method and detailing the methodology have been published (*4*−*13*). Here, the standard unnatural amino acid methodology is outlined, significant applications of the approach are summarized, and recent major improvements in the method are discussed.

Methodology

Incorporating unnatural amino acids into proteins using nonsense codon suppression requires four fundamental reagents (Figure 1): (1) an unnatural amino acid of interest; (2) a suppressor tRNA charged with the unnatural amino acid and containing an anticodon that recognizes a nonsense codon; (3) DNA coding for the protein of interest with a nonsense codon at the desired site of unnatural amino acid incorporation; (4) a translation system containing ribosomes. Following is a more detailed description of each of these reagents.

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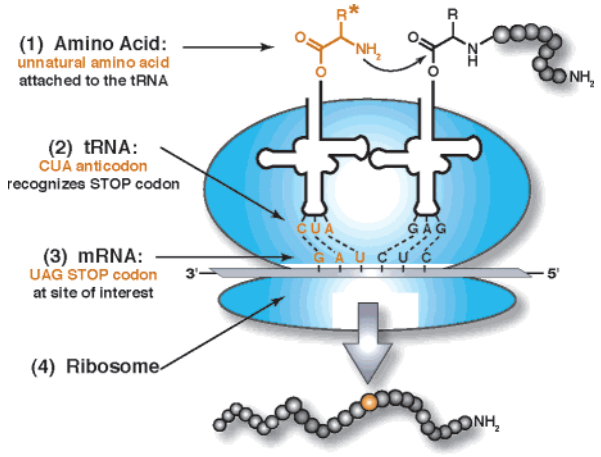


FIGURE 1: Summary of the key reagents required for incorporating unnatural amino acids into proteins using nonsense codon suppression.

The Unnatural Amino Acid

Over 100 different unnatural residues have been introduced into proteins using the nonsense suppression method (Chart 1). A survey of these residues suggests that, with the exception of D-amino acids (*4*, *14*, *15*), there are no absolute limitations in terms of what types of structures can be incorporated into proteins using this approach. On the other hand, the overall suppression efficiency (i.e., the efficiency of unnatural amino acid incorporation) is influenced in part by the identity of the amino acid. For example, in most cases suppression of nonsense codons with α-hydroxy acids is more efficient than suppression with the corresponding α-amino acids (*16*−*19*). This may result from the increased stability of hydroxyacylated tRNAs relative to aminoacylated tRNAs (*20*−*23*). In addition, nonpolar residues appear to suppress better than charged residues (*15*, *24*, *25*). Finally, residues with branching at the α- or *β*-carbons or both do not suppress, as well as residues without branching or with branching farther removed from the α-carbon (*15*, *26*). These "rules" appear to vary depending on the translation system, the identity of the protein, the suppression site, and the suppressor tRNA and should, therefore, only be considered as guidelines. In most of the unnatural amino acid studies conducted to date, the unnatural amino acids have been activated and coupled to the suppressor tRNA exogenously through a combination of chemical and enzymatic synthesis (*4*, *6*, *18*, *27*, *28*).

The Nonsense Suppressor tRNA

Aminoacylated tRNAs containing an anticodon that "reads" nonsense codons are so-called nonsense suppressor tRNAs. These tRNAs must satisfy two criteria to be effectively used to incorporate unnatural amino acids into proteins. First, the nonsense suppressor tRNA must be efficiently recognized by the protein biosynthetic machinery. Second, the nonsense suppressor tRNA must be orthogonal; that is, it must *not* be a substrate for endogenous aminoacyl tRNA synthetases (aaRS) within the translation system. If the nonsense suppressor tRNA is not orthogonal, it will be charged with *natural* amino acids and, as a result, mixtures of protein products that cannot be functionally or physically separated from one another will be produced. Several orthogonal suppressor tRNAs have been developed and utilized to efficiently incorporate unnatural amino acids into proteins (*25*, *29*−*34*). In most of the unnatural amino acid studies conducted to date, the suppressor tRNA has been charged with unnatural amino acids through a series of chemical and enzymatic steps. In particular, the unnatural amino acid is first chemically activated and coupled to a synthetically prepared dinucleotide (pdCpA).[1] The resulting aminoacylated dinucleotide (aa-pdCpA) is then enzymatically ligated to a 74 base-pair suppressor tRNA lacking the terminal pCpA dinucleotide to produce full-length (76 base-pair) aminoacylated suppressor tRNA (*4*, *6*, *27*).

The DNA

Nonsense codon suppression typically uses a noncoding triplet (usually the amber stop codon "UAG") to code instead for an unnatural amino acid. In particular, the coding triplet for the protein residue of interest is replaced with a nonsense codon using conventional site-directed mutagenesis. Suppression of the nonsense codon with an aminoacylated tRNA containing the appropriate anticodon results in the incorporation of an amino acid at that site. The "UAG" nonsense codon has been the most widely utilized for nonsense suppression but orthogonal tRNAs recognizing the other natural nonsense codons (UAA, UGA) as well as four and five base pair codons have also been investigated (*9*, *35*−*41*). Developing alternative nonsense codons has facilitated site-specifically incorporating two different unnatural amino acids into the same protein (*42*−*44*).

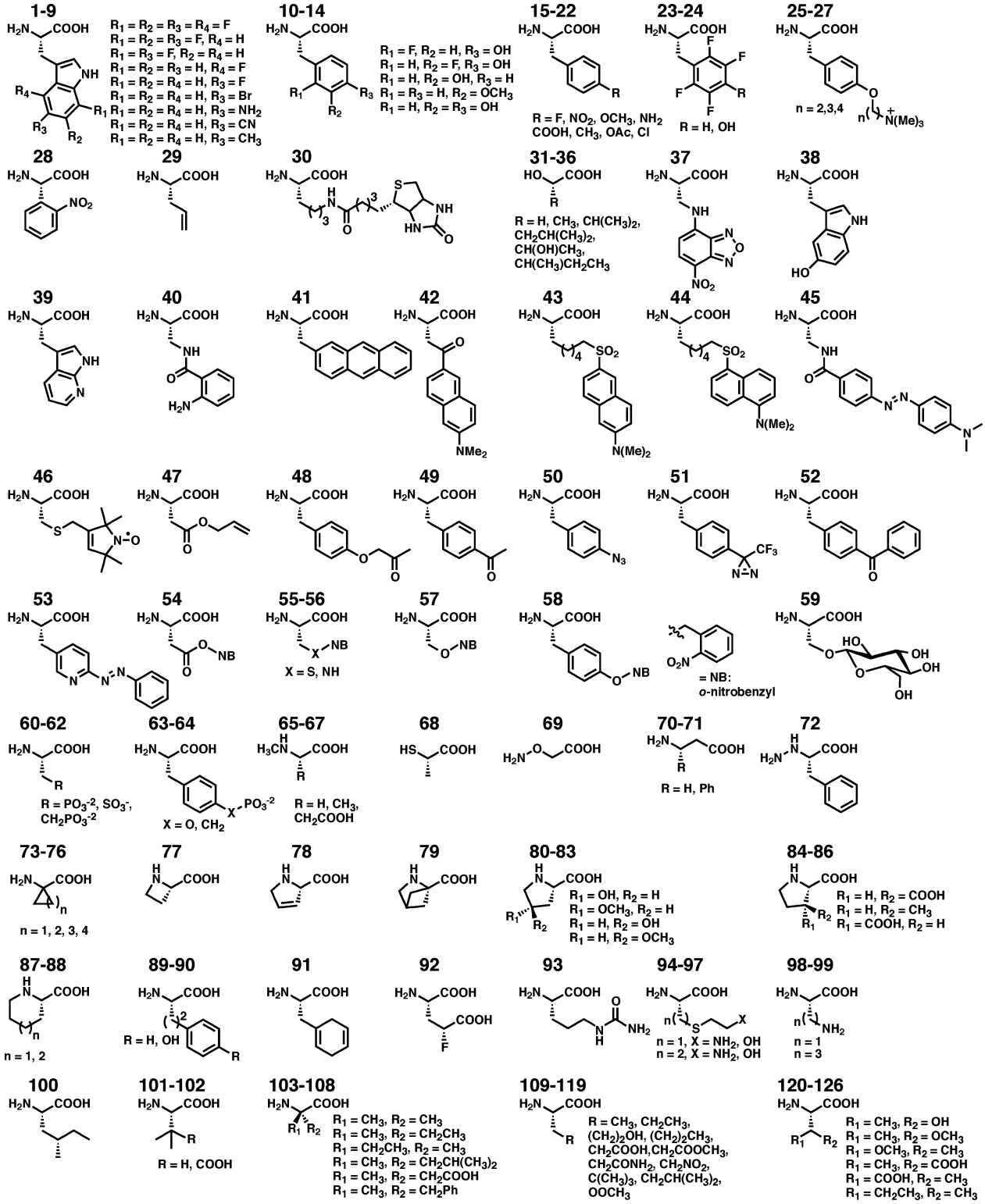
The Translation System

The translation system is comprised of the remaining components required for protein synthesis (ribosomes, natural tRNAs, natural tRNA synthetases, natural amino acids, ATP, etc.). Nonsense suppression has thus far been achieved in two contexts, namely, cell extracts and intact cells. The most widely utilized cell-extract translation systems include lysates from *Escherichia coli*, rabbit reticulocytes, and wheat germ. Simply mixing the cell extract with aminoacylated suppressor tRNA and DNA containing the nonsense codon at the site of interest provides for the synthesis of full-length protein containing the unnatural amino acid.

In 1995, the efforts of Lester, Dougherty, and co-workers led to the first example of using nonsense codon suppression to incorporate unnatural amino acids into proteins expressed in intact cells. In particular, unnatural amino acids were incorporated at several sites in an ion channel heterologously expressed in *Xenopus* ooctyes (*45*). These cells have proven to be a particularly well-suited translation system for studying transmembrane proteins, such as ion channels, using unnatural amino acid mutagenesis. First, *Xenopus* oocytes (∼1 mm diameter) are easily microinjected with aminoacylated suppressor tRNA and mRNA. Second, the oocytes not only efficiently synthesize, fold, assemble, and insert full-length protein into the plasma membrane but also effectively purify the protein since truncated translation products generally do

[1] Abbreviations: tRNA, transfer ribonucleic acid; DNA, deoxyribonucleic acid; mRNA, messenger ribonucleic acid; aaRS, aminoacyl-tRNA synthetase; pCpA, 5′-*O*-phosphorylcytidylyl(3′-5′)adenosine; pdCpA, 5′-*O*-phosphoryl-2′-deoxycytidylyl(3′-5′)adenosine; nAChR, nicotinic acetylcholine receptor.

Chart 1: Sturctures of Unnatural Residues Incorporated into Proteins Using Nonsense Codon Suppression



not get inserted into the plasma membrane. Third, the standard assay for characterizing ion channel function, electrophysiological recordings of ionic currents from single cells, is exquisitely sensitive requiring only femtomoles of protein ($\sim 10^7$ ion channels) per cell. Finally, the synthetic organic chemistry required to prepare the aminoacylated dinucleotide (aa-pdCpA) for coupling to the suppressor tRNA becomes a one-time effort because only nanograms of the aminoacylated suppressor tRNA are required for each study.

Applications

The nonsense suppression method for site-specific incorporation of unnatural amino acids into proteins has been applied to both soluble and transmembrane proteins to address a number of fundamental structure−function questions. Described below are several studies demonstrating the broad utility of the method. Included are examples where a conventional approach might have been used to address the

same question. In these cases, the power of unnatural amino acid mutagenesis lies in providing a comparatively more precise means of approaching the particular question. Other examples serve to highlight the capacity of the method to manipulate proteins in ways that simply cannot be achieved using conventional approaches.

Identifying Structural and Functional Domains

Unnatural amino acid mutagenesis has been effectively used in a number of studies to provide insight into various structural and functional protein domains. For example, direct evidence for the occurrence of a cation−π interaction between a neurotransmitter (acetylcholine) and its receptor (the nAChR) came from an elegant unnatural amino acid mutagenesis study. Cation−π interactions are favorable noncovalent interactions between cationic species, such as quarternary and protonated amines found in many neurotransmitters, and the electrostatic potential on the face of aromatic rings, such as tryptophan residues (*46*). Cation−π interactions had been proposed to underlie the association of acetylcholine, a quarternary amine, with the ligand binding domain of the nAChR, a region of the protein rich in aromatic residues (*47*). To evaluate this hypothesis, unnatural amino acid mutagenesis was used to replace individual tryptophan residues within the nAChR binding site with a series of tryptophan analogues substituted with various electron-withdrawing groups (Chart 1, **1**−**9**) (*48*). A cation−π interaction between the ligand and the receptor at α-Trp^{149} was clearly identified on the basis of shifts in the dose−response relationship that predictably correlated with the electrostatic potential of the tryptophan indole ring. Notably, the crystal structure of an acetylcholine binding protein (ACHBP) confirmed the assignment of Trp^{149} as a key ligand binding element. Many proteins are proposed to have cation−π interactions, and unnatural amino acid mutagenesis is an unsurpassable technique for testing these hypotheses (*49*).

In cases where little is known about the overall structure of the protein (e.g., membrane topology, disulfide connectivity) or the function of particular protein regions (e.g., C- and N-terminal domains), unnatural amino acid mutagenesis has also proven to be an informative technique. For example, a photoreactive residue (Chart 1, **28**) was developed to enable site-specific peptide backbone cleavage with ultraviolet light, a biotin-containing residue (Chart 1, **30**) was created to chart protein membrane topology, and α-hydroxy acids (Chart 1, **31**−**36**) were constructed to map disulfide connectivity (*17*, *50*, *51*).

Identifying Conformational Changes

In addition to providing insight into the static structure of proteins, unnatural amino acid mutagenesis has also proven to be well-suited for investigating protein dynamics. The incorporation of unnatural residues that result in the introduction of novel backbone structures into proteins has been developed to probe the role of individual hydrogen bonds in protein stability and activity (*16*, *52*, *53*). For example, α-hydroxy acids (Chart 1, **31**−**36**) have been used to map activity-dependent changes in protein secondary structure (*19*). The substitution of an α-amino acid with an α-hydroxy acid leads to an amide-to-ester mutation in the protein backbone, removing a hydrogen bond donor at the site of incorporation. Processes that involve conformational changes in secondary structural elements that rely on backbone hydrogen bonds (e.g., α-helicies, β-sheets) are then revealed by changes in the relative stability of the protein in different states (e.g., the open versus closed state of an ion channel).

The incorporation of unnatural amino acids containing fluorescent side chains (Chart 1, **37**−**45**) provides another approach for monitoring protein conformational changes. A variety of fluorescent unnatural amino acids have been incorporated into proteins using the nonsense suppression approach (*26*, *36*, *43*, *44*, *54*−*56*). In one study, the incorporation of two different fluorescent amino acids into the protein dihydrofolate reductase provided a means of monitoring protease-mediated cleavage of the protein into two fragments using fluorescence resonance energy transfer (FRET) (*44*).

Identifying Protein−*Protein Interactions*

Unnatural amino acid mutagenesis also provides a means of identifying protein−protein interactions. While there are a number of conventional approaches for identifying such interactions (e.g., affinity chromatography, immunoprecipitation of protein complexes from cell lysates, yeast-two-hybrid screens), these methods are often hindered by relatively weak associations between interacting proteins. The incorporation of unnatural amino acids bearing cross-linkable side chains into proteins provides a complementary approach. The most common types of photo-cross-linkers (i.e., benzophenones, aryl azides, diazarines) become reactive radicals when irradiated with ultraviolet light. The radical species can undergo various insertion reactions with associated proteins forming covalent adducts. Unnatural amino acids bearing each of these reactive groups in their side chains (Chart 1, **49**−**51**) have been successfully incorporated into proteins and, in a few cases, used to identify protein−protein interactions (*26*, *57*−*61*).

Unnatural amino acid mutagenesis also provides a means of characterizing phosphorylation-dependent protein−protein interactions. For example, the incorporation of caged hydroxyl side chains (Chart 1, **57** and **58**) provides a means of blocking a particular phosphorylation event until the cage is removed with ultraviolet light. A series of phosphorylated residues, including several phosphatase-resistant analogues, have also been designed to investigate phosphorylation-dependent signaling (Chart 1, **60**−**64**). The development of caged analogues of such phosphorylated residues will allow for an unparalleled level of temporal control over phosphorylation-dependent signaling.

Limitations and Improvements

The site-specific incorporation of unnatural amino acids into proteins has clearly proven to be a powerful tool for probing protein structure and function. Still, the number of laboratories implementing this approach is not commensurate with its utility. The primary limitations of the method and the steps being taken to overcome them are discussed below.

One of the main reasons that unnatural amino acid mutagenesis has not become a standard laboratory tool is that implementing the approach requires the combined expertise of a molecular biologist, a protein biochemist, and

a synthetic organic chemist. In truth, "expertise" is the wrong word—the molecular biology is conventional site-directed mutagenesis, the biochemistry relies on standard protein purification tools, and the organic synthesis is thoroughly documented in the literature. The real difficulty lies in simultaneously gathering each of these techniques in one laboratory.

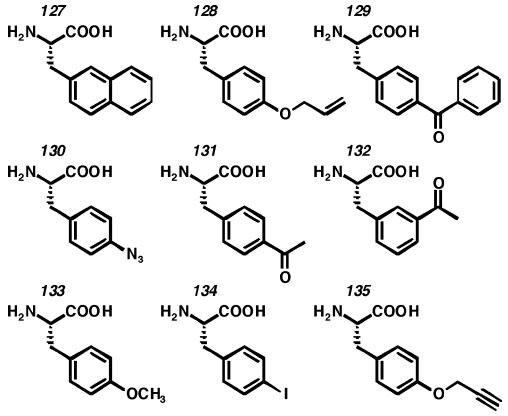
Another limitation associated with unnatural amino acid mutagenesis is the amount of protein that can be conveniently obtained using nonsense codon suppression. Since the aminoacylated tRNA is a stoichiometric reagent, the amount of full-length protein produced is limited by the amount of nonsense suppressor tRNA that can be prepared and introduced into the translation system. This is a particularly relevant concern among biochemists wishing to conduct structural studies.

Two recently developed in cell translation systems simultaneously address both of these limitations, providing a significant advance in the methodology. In particular, Schultz and co-workers have made a conceptual leap in developing a method for charging nonsense suppressor tRNAs with unnatural amino acids within *E. coli* and *Saccharomyces cerevisiae*, eliminating the need to chemically synthesize and subsequently introduce the aminoacyl suppressor tRNA into the cells (*62*, *63*). Aminoacylation of the suppressor tRNA is carried out enzymatically within cells by tRNA synthetases that have been reengineered to specifically recognize and couple the unnatural amino acid of interest (and not any other amino acids) to the suppressor tRNA (and not any other tRNAs). This approach requires that both the aaRS and the nonsense suppressor are orthogonal within the translation system. That is, the aaRS must recognize *only* the nonsense suppressor tRNA as a substrate, and conversely, the nonsense suppressor must be recognized by only this aaRS. The in cell aminoacylation/translation system also solves the issue of quantity. Cells expressing the orthogonal aaRS/tRNA pair can be grown on any scale in media supplemented with the unnatural amino acid of interest to produce milligram-per-liter quantities of suppressed protein.

It is worth acknowledging that an obvious *theoretical* limitation associated with achieving nonsense codon suppression in a cellular context is that the standard nonsense codon (UAG) is also used as a "stop" signal in many proteins expressed in cells. In principle, the nonsense codon suppression methodology should be limited to in vitro translation systems, which contain only the transcript for the protein of interest. Yet, unnatural amino acid mutagenesis has been successfully accomplished in both single cells and single-celled organisms. Perhaps most notably, the expression level (~2.0 mg/L) of proteins containing unnatural amino acids that have been incorporated using the in cell aminoacylation/translation approach is often comparable to the expression level of the corresponding wild-type protein expressed in the same media.

The in cell aminoacylation/translation approach, while obviating the need for organic synthesis if the amino acid of interest is commercially available, requires a substantial amount of molecular biology. Specifically, an orthogonal aaRS/tRNA pair must be designed for each unnatural amino acid using an iterative series of mutations and genetic screens. To date, several orthogonal aaRS/tRNA pairs have been developed to introduce primarily phenylalanine analogues

Chart 2: Unnatural Amino Acids Incorporated into Proteins Expressed in *E. coli* and *S. cerevisiae* Using the in Cell Aminoacylation/Translation System



into proteins expressed in *E. coli* and *S. cerevisiae* (Chart 2) (*59*, *61*−*71*). In principle, this approach can be extended to any unnatural amino acid of interest.

A number of the unnatural amino acids that have been introduced using the in cell aminoacylation/translation approach will likely prove to be of general utility. For example, the two photo-cross-linkers (Chart 2, **129** and **130**) provide a means of identifying protein−protein interactions among full-length, functional proteins expressed in intact cells. In addition, the ketone-containing residues (Chart 2, **131** and **132**) provide a means of site specifically tagging proteins with any one of a number of molecules. In aqueous solution, the keto group reacts with hydrazide and alkoxyamine derivatives to form hydrazones and oximes, respectively. The products are stable under physiological conditions and are formed selectively in the presence of other functional groups present in proteins. This approach has been successfully used to site-specifically label proteins in cells with fluorophores (*72*, *73*). The azido and the acetylene amino acids (Chart 2, **130** and **135**) provide a complementary approach for tagging proteins with biophysical probes. These amino acid side chains can be covalently linked to molecules containing either an alkyne or an azide using a [3 + 2] cycloaddition reaction. This approach was also used to introduce a series of dyes into a protein (*69*). The in cell aminoacylation/translation strategy for incorporating unnatural amino acids into proteins represents a significant breakthrough in the field that will undoubtedly facilitate additional laboratories using unnatural amino acids to study proteins.

A third limitation associated with unnatural amino acid mutagenesis is that of context. Nearly all of the unnatural amino acid studies reported thus far have utilized the *Xenopus* ooctye, *E. coli*, or *S. cerevisiae* translation systems. To be sure, these are excellent systems for addressing a number of structure−function questions. Nonetheless, many biological questions cannot be convincingly addressed in these contexts, and expanding the technology to other cell types and multicellular organisms will encourage additional applications of the method. The appropriate cellular context is an important issue, for example, in studies focused on mammalian signal transduction cascades. Initial efforts to extend the methodology to mammalian cells have met with some success (*74*−*76*). In particular, orthogonal nonsense suppressor tRNAs have been identified and successfully intro-

duced at stoichiometric levels into mammalian COS1 cells using the transfection reagent Effectene (Qiagen) and in CHO cells and cultured rat hippocampal neurons using microelectroporation (*74*−*76*). Further, the CHO cell experiments demonstrated that unnatural amino acids could be site-specifically incorporated into an ion channel expressed in these cells (*76*).

Over the course of the past decade, tremendous progress has been made in both developing and applying the unnatural amino acid mutagenesis methodology to studying protein structure and function. The future will likely see many more investigators embracing this approach to manipulate proteins as it realizes its promise of becoming a tool with enormous potential.

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