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Laboratory Experiment

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Utilizing Unnatural Amino Acids To Illustrate Protein Structure–Function Relationships: An Experiment Designed for an Undergraduate Biochemistry Laboratory

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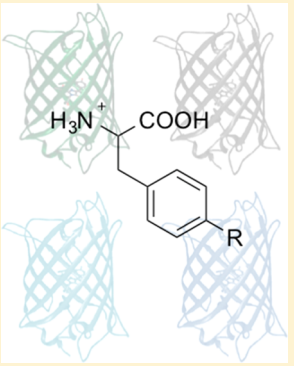
Supporting Information

ABSTRACT: The site-specific introduction of unnatural amino acids (UAAs) has been demonstrated to be a useful tool in protein engineering. Moreover, the incorporation of a UAA into a protein has become feasible with the increased commercial availability of UAAs and robust expression plasmids. In addition to the ease of incorporation, the concepts utilized in the incorporation of a UAA are ideal to illustrate key concepts involved in protein translation and structure–function relationships. Consequently, a novel experiment has been developed for the incorporation of various UAAs into the GFP fluorophore to alter spectral properties. This experiment provides students with a hands-on approach for transformation, protein expression and purification, and structure–function analysis.



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A learning outcome in most biochemistry courses is instilling an understanding of protein structure–function relationships. To master this concept, students must be able to recognize how changing even a single amino acid in the protein's primary sequence directly impacts the structure of the protein. Moreover, linked to this concept is the central dogma, a core tenant of any molecular biology or biochemical program. To understand how alterations in a protein's primary sequence arise, students must recognize that the genetic material coding each amino acid of a protein is stored in the DNA. This DNA then gets transcribed into RNA, which is ultimately translated into the amino acid sequence. Thus, an understanding of protein structure and function is intrinsically tied to an understanding of the central dogma. That being said, it is often difficult to illustrate this connection in a purely classroom setting. Consequently, we have developed a laboratory experiment to highlight these connections simultaneously.

Unnatural amino acids (UAAs) represent a unique means to convey all of these concepts.[1,2] In particular, the ability to introduce novel chemical functionality unavailable in the 20 canonical amino acids allows students to observe how altering amino acids can affect the microenvironment around an amino acid residue, drastically affecting how the protein folds and behaves. This represents an advantage over simply changing one naturally occurring amino acid for another, as these changes tend to be more nuanced and thus make it harder for students to fully comprehend the chemical alteration they have performed. Furthermore, utilization of the Schultz methodology of UAA incorporation, whereby successful incorporation of an UAA hinges on the suppression of a TAG point mutation, encourages students to engage with the transcription and translational processes (Figure 1).[1−3] In doing so, they can better visualize how mutations at the DNA level are carried through into a protein's final structure and function. Moreover, because of the necessity to introduce orthogonal translational machinery (a tRNA and aminoacyl tRNA synthetase pair capable of recognizing and charging the UAA to the tRNA), the experiment also provides a mechanism to introduce multiple molecular cloning techniques to the students.[3] Finally, the use of UAAs in biological chemistry is becoming increasingly prevalent in multiple applications (therapeutics, bioconjugates, protein probes, etc.), and thus exposing students to this valuable technique has pedagogical advantages beyond the scope of the experiment.

Herein, we report a laboratory exercise that uses the Schultz methodology to incorporate commercially available UAAs into Tyr66 of green fluorescent protein (GFP; Figure 2). This tyrosine, along with Ser67 and Gly68, is essential in fluorophore formation and thus is directly linked to protein function.[4,5] Consequently, students can directly visualize the alteration in UAA structure as a change in the GFP fluorescence spectrum, illustrating how protein function is altered by relatively minor changes to the amino acid sequence. Furthermore, compared to other methods to illustrate structure–function relationship

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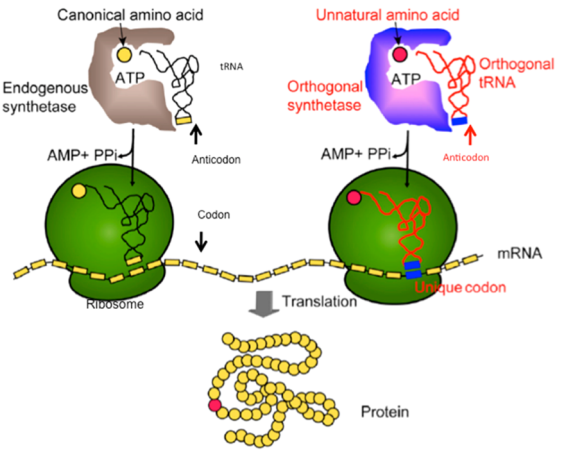

Figure 1. Mechanism for genetic incorporation of UAAs. An orthogonal synthetase must be introduced that does not cross-react with any natural tRNA or amino acid. This is coupled with a new tRNA that harbors an anticodon for the TAG codon. When the ribosome encounters the TAG codon, a new amino acid is incorporated rather than the discontinuation of translation. Adapted from *Chem. Biol.* **2009** 16, 323−336.

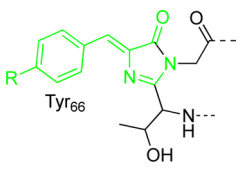

Figure 2. Structure of GFP fluorophore. Tyrosine-66 (green) will be replaced with different UAAs to alter spectral properties.

using GFP, such as PCR mutagenesis, the Schultz' methodology employing UAAs is more direct and requires less genetic manipulation on the part of the student given the time constraints of a laboratory setting.[5,6] As a result, it is more likely to produce a functional mutated-protein product and therefore concretely illustrates for the students the effects of altering even a single amino acid on protein function.

LEARNING OBJECTIVES

The utilization of UAAs is a cutting edge technology and is an excellent mechanism to foster discussion involving protein structure−function relationships. To the best of our knowledge, there has been no reported undergraduate teaching lab incorporating UAA technology.[7,8] As a result, we sought to generate a simple and inexpensive laboratory exercise that would introduce students to this rapidly expanding technology while simultaneously reinforcing the principles of protein structure and function and the central dogma. Moreover, this experiment confers practical experience in bacterial transformation, protein expression, protein purification, and fluorescence spectrometry. The experiment also seeks to solidify key concepts from the classroom, specifically involving translational processes.

EXPERIMENTAL OVERVIEW

In this experiment, the expression and purification of an UAA-containing GFP as well as a wild type GFP will be performed. While typically a unique aminoacyl-tRNA synthetase (aaRS) must be evolved for a specific UAA, recent reports have demonstrated a unique aaRS, the *p*CNF aaRS, that is capable of incorporating up to 20 different UAAs.[9] This is optimal for this experiment to minimize the storage, purification, and organization of multiple aaRS plasmids. The other requirement for UAA incorporation is a gene containing a TAG codon at the residue desired for UAA incorporation, allowing for the suppression of the stop codon by UAA integration if the system is properly functioning. Using plasmids harboring the previously reported promiscuous aaRS, *p*CNF aaRS, and GFP_{TAG66}, students will perform a cotransform of competent *E. coli*. This can be accomplished by either electroporation or chemical transformation with competent cells, facilitating discussions on mechanisms of bacterial transformation, transformation efficiency, and the requisite antibiotics to facilitate plasmid uptake and maintenance. The transformed cells will then be used to induce GFP expression in the presence of an assigned UAA. Depending on laboratory size, each pair of students may be assigned a different commercially available UAA. Additionally, students will transform and express wild-type GFP for comparison of the fluorescence emission profile of their UAA-containing GFP versus the wild-type. All plasmids required for the experiment are available from Addgene (Plasmid #48215) or can be obtained from the authors, and the UAAs are all commercially available.

Protein purification may be carried out by numerous methods. Hydrophobic interaction columns, which are commercially available and simple for the undergraduate student, will be used to partially purify both UAA-containing GFP and wild-type GFP. Our results indicate that this procedure affords GFP in good yield with only minor contaminants and with approximately no effect on the fluorescence profile of the protein.[9]

This experiment will incorporate several key biochemistry skills for the undergraduate researcher including electroporation, protein expression and purification, fluorimetry, and UAA-mutagenesis.

EXPERIMENTAL PROCEDURE

Laboratory Setup and Procedure

A wild-type GFP transformation and expression protocol was adapted from the commercially available pGLO plasmid kit (Biorad; Cat # 166−0003EDU).

UAA-mutagenesis using the Schultz methodology requires the preparation of plasmid stocks harboring the pEVOL-*p*CNF-aaRS plasmid with chloramphenicol-resistance, the pET-GFP_{WT} plasmid with ampicillin-resistance, and the pET-GFP_{TAG66} plasmid with ampicillin-resistance. Additionally, 100 mM stocks of *p*-azidophenylalanine (*p*AzF), *p*-biphenylphenylalanine (*p*BiF), *p*-acetylphenylalanine (pAcF), and *p*-iodophenylalanine (*p*IF) in deionized water should be prepared prior to the start of the laboratory period (see Supporting Information).

Students work in pairs over the course of 3 weeks, and the work typically requires 2 h for each lab meeting. Week 1 of the experiment focuses on bacterial transformation and mutant GFP expression. Week 2 involves both wild-type and mutant GFP purification and fluorescence. Week 3 entails a "GFP symposium" facilitating discussion about the techniques utilized in lab and student presentations.

Week 1. In the first week, students perform transformations and induce expressions of GFP mutants harboring an unnatural amino acid. The chemical transformation of pET-GFP_{WT} is performed in direct accordance with the Bio-Rad pGLO kit (substituting 1 mM IPTG for arabinose in the agar plates). This

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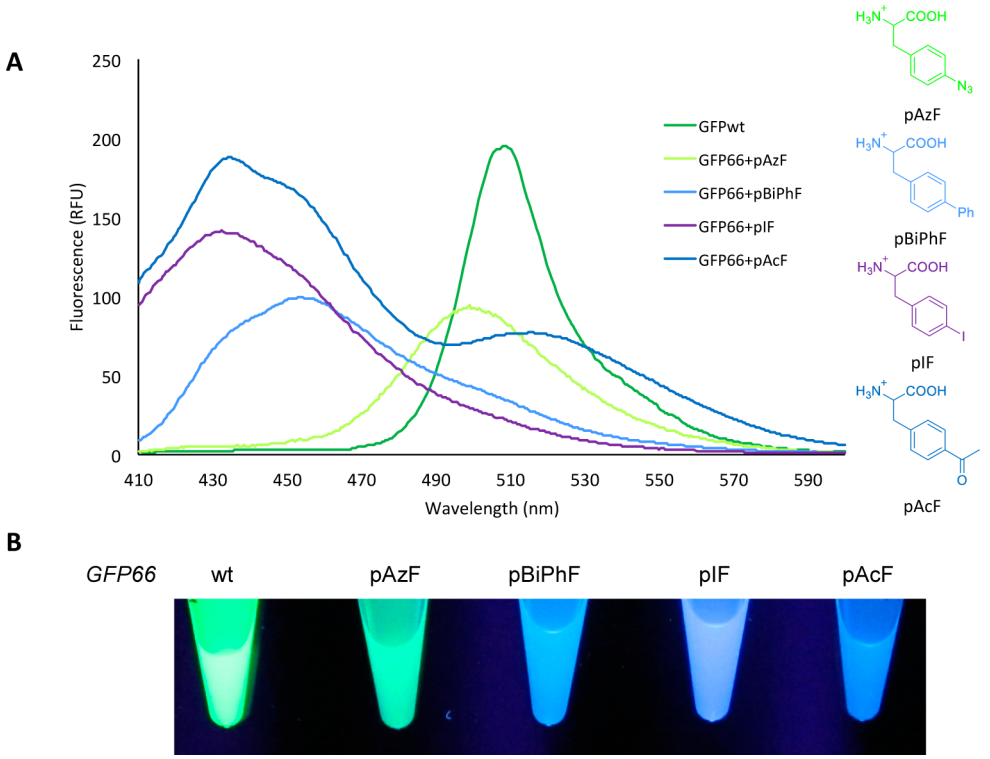

Figure 3. Fluorescence results for the expression of wild type GFP and GFP_{TAG66} mutants harboring a UAA. (A) Fluorescence spectra obtained on a fluorimeter exciting at 395 nm and scanning emission from 410−600 nm. (B) Visualization of mutant protein fluorescence on a transluminator with an excitation wavelength of 365 nm.

plasmid encodes the wild-type GFP and does not require the aaRS machinery. The plasmid can also be transformed by electroporation to provide a direct comparison to the chemical transformation. This portion of the lab emphasizes the transformation of plasmid DNA into *E. coli* for heterologous expression. If available, students may also perform an optional double transformation using 0.5 μL of pEVOL-*p*CNF plasmid and 0.5 μL of pET-GFP_{TAG66} plasmid, both at approximately 100 ng/μL with an electroporator at 1800 V. These transformations should be recovered at 37 °C for 30 min, then plated on plates containing ampicillin and chloramphenicol.

While transformations are being performed, students also induce the expression of a mutant GFP with their specified UAA using a second bacterial culture that was previously prepared. Two days prior to the experiment, the instructor must cotransform the pEVOL-*p*CNF plasmid and pET-GFP_{TAG66} as described above. The next day, a colony should be selected and grown in 10 mL of LB Media containing ampicillin and chloramphenicol at 37 °C overnight. The day of the lab, approximately 1−2 h before the scheduled time, a 10 mL expression culture of the cells should be inoculated for each group at OD_{600} 0.1. During the actual experiment, students will begin expressing their UAA-containing GFP using an expression culture that has grown to log phase (OD_{600} = 0.6−0.8). Expression of the GFP_{TAG66} is induced with the addition of 10 μL each of 1000× stocks of IPTG and 20% arabinose. Also, at this time, students add 100 μL of their assigned 100 mM UAA stock. This laboratory period facilitates a discussion of typical transformation procedures (students are exposed to both heat shock and electroporation in the exercise) as well as a discussion of molecular genetics for gene expression. Finally, the use of the Schultz methodology for incorporation of the UAA into GFP_{TAG66} facilitates a discussion of gene transcription and protein translation.

The next day, the laboratory supervisor centrifuges the expression cultures of the UAA-containing GFP (5000*g* for 10 min). The cell pellets are then stored at −80 °C until the next laboratory session. Additionally, at some point in-between lab periods, students observe the fluorescence of the *E. coli* cell pellet and count the colonies on their transformation plates. This allows them to identify the differences between electroporation (UAA plasmids, and possibly the wild-type plasmid) and heat-shock (wild-type plasmid) as well as single and double transformations. These colony counts are used to calculate the transformation efficiency for the single transformations versus the double transformations.

Week 2. In the second week, students use hydrophobic interaction columns (HICs) to purify both their wild-type and UAA-containing GFP. This week follows the protocol described in the Green Fluorescent Protein Chromatography Kit (Bio-Rad; Cat # 166−0005EDU). By using their partially purified protein at a concentration of 0.5−1 mg/mL, students prepare a sample for fluorescence detection by diluting 10 μL of their protein in 3 mL of 1× PBS. Students then observe the fluorescence spectra of their wild-type and UAA-containing GFPs using a fluorimeter set to excite at 395 nm and scan between 410 and 600 nm.

This week also has the potential to facilitate a discussion on protein purification techniques, as the HIC process pulls down many contaminating proteins along with the UAA-mutated GFP. As such, column fractions can be analyzed via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with coomassie blue to highlight the limitations of this purification technique (see Supporting Information). Furthermore, if time is not available to stain

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Table 1. Comparison of Standard GFP Laboratories versus the UAA Laboratory

Learning Outcomes for Standard GFP Transformation Experiment	Learning Outcomes for UAA-Mutagenesis of GFP Experiment
• Basic understanding of transformation techniques as well as protein purification (which obviously pairs well with protein properties)	• All those listed in the "Standard" GFP Transformation Experiment
• Provides student with an opportunity to understand selection processes in conjunction with plasmid design (i.e., antibiotic resistance as a means to ensure plasmid maintenance)	• Facilitates discussion of translational processes via the Schultz' methodology for UAA incorporation
• Provides an illustration of molecular genetics by providing a means to discuss operators and promoters	• Facilitates discussion of protein structure−function relationship, as the incorporation of different UAAs alters the GFP-chromophore, affecting fluorescence profile
	• Provides students with the opportunity to practice use of a fluorimeter, a standard laboratory instrument in most chemical settings; also facilitates a discussion of fluorescence, a topic typically relegated to physical chemistry
	• Provides students with exposure to current technologies (e.g., UAA incorporation) that are widely employed within the field of chemical biology

and destain with coomassie blue, the GFP fluorescence can be directly observed through the SDS-PAGE gel using a UV transluminator or lamp.

Week 3. In the third week, students present their results as well as a discussion of topics related to protein expression, purification, and fluorescence. Each student is assigned a specific topic on which to prepare a 5−10 min presentation. Examples of topics include biological relevance of lysozyme, methods of cell lysis, DNA mutagenesis for the introduction of the TAG codon, aaRS evolution, mechanism of chemical transformations, antibotics and antibiotic resistance, protein induction by IPTG/Arabinose, vectorology, the chemical basis of GFP fluorescence, and numerous other topics related to the experiment. Additionally, the results of the experiment are discussed and compiled fluorimetry data are presented to truly illustrate the effect of the different amino acids on GFP fluorescence

HAZARDS

E. coli are a BSL-1 organism, the appropriate precautions should be taken, and the experiment requires IBC approval. Precautionary measures should be taken when analyzing protein samples under a UV lamp at 365 nm. The main chemical hazard is acrylamide, which is an irritant, and potential neurotoxin and should be handled with care (see Supporting Information for complete list of chemical precautions).

RESULTS AND DISCUSSION

Overall, the experiments described above translated well in a laboratory setting. While the traditional pGLO GFP lab has been employed in our advanced biochemistry laboratory for the past several years, the UAA component has been included in the past two years. The first year involved nine students in the lab section with 12 students the second year, for a total of 21 student participants. The double transformations that the students performed yielded approximately five colonies per lab group, which is to be expected of double transformations. Single transformations afforded much higher transformation efficiencies than those referenced in the literature and ranged from $0-10^9$ cfu. The efficiencies were typically student-dependent based on previous experience and their ability to follow directions (see Supporting Information for a complete list of pitfalls). These results promote discussions into antibiotic resistance and requisites for colony growth on antibiotic plates. Protein expression levels were robust, the GFP_{WT} and the UAA-containing GFP_{66} both yielding between 0.5 and 1 mg/mL of purified protein (see Figure S1).

The UAA-mutagenesis of the GFP_{66} was successful for all UAAs used in this laboratory exercise, as demonstrated by the successful shift in the UAA-containing GFP_{66} relative to the GFP_{WT}. Gratifyingly, spectral shifts of GFP fluorescence were observed for each mutant, demonstrating the relationship between protein structure and function, and the influence of a single amino acid mutation (Figure 3). The most dramatic blue shift was observed for the pAcF mutant, most likely due to the resonance into the carboxylic oxygen. Overall, students were able to effectively transform bacteria with the plasmids as well as express and purify functional protein and analyze its spectral properties.

This experiment was conducted in an upper-level biochemistry lab targeted for upper-division students with a background in the subject. Compared to a standard protein expression/purification lab, in which students transform GFP_{WT} into *E. coli* and subsequently purify the protein, this exercise provides students with exposure to considerably more biochemical and molecular biology techniques. In particular, the use of the Schultz methodology for UAA incorporation allowed students to engage with topics from lecture, such as translation and protein structure−function relationships, in a way that was not accessible via a more traditional lab (Table 1). On the basis of student evaluations of the experiment, an appreciation of how the experiment concretely illustrates protein structure−function relationships was the most powerful learning outcome. Moreover, the use of the fluorimeter allowed students to visualize the impact of their UAA incorporation, thereby reinforcing how even a single amino acid alteration can drastically impact a protein's function. This concept is perfectly illustrated by the integration of UAAs into the laboratory and can easily be achieved over the course of two lab meetings.

The experiment also provides practical introductions to bacterial transformations and illustrates the differences in transformation efficiency between various protocols. Additionally, the necessity for double-transformations facilitates discussions for mechanism of antibiotic resistance and the necessity of antibiotic resistance onto the plasmids to facilitate bacterial selections. Finally, key aspects of fluorimetry are also introduced in the experiment as differing functional groups result in spectral shifting of the fluorophore emission.

Upon completion of the experiment during week 3, the learning outcomes were assessed both by presentation projects and by an evaluation form (see Supporting Information). Students were asked to distill the big picture ideas as well as assess the effectiveness of the laboratory. Overall, students were overwhelmingly positive in the assessment of the lab, and all of the students were able to clearly and concisely communicate both the purpose of the lab and fundamental concepts about plasmids and protein translation. Finally, learning outcomes were assessed by the completion of a typical laboratory report

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summarizing the experiment and the overall results for each student.

SUMMARY

UAAs are an excellent mechanism for the study of protein structure−function relationships. This developed experiment for an upper division biochemistry course provides a simple means of demonstrating this feature of UAAs. Moreover, the mechanism of UAA incorporation facilitates substantial learning and solidifies several key concepts including bacterial transformations, molecular cloning, and fluorescence.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.5b00627.

Student experimental handout; instructor notes; list of chemicals (PDF, DOCX)

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Notes

The authors declare no competing financial interest.

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