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Expression, Purification, and Analysis of Unknown Translation Factors from *Escherichia coli*: A Synthesis Approach*

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New approaches are currently being developed to expose biochemistry and molecular biology undergraduates to a more interactive learning environment. Here, we propose a unique project-based laboratory module, which incorporates exposure to biophysical chemistry approaches to address problems in protein chemistry. Each of the experiments described herein contributes to the stepwise process of isolating, identifying, and analyzing a protein involved in a central biological process, prokaryotic translation. Students are provided with expression plasmids that harbor an unknown translation factor, and it is their charge to complete a series of experiments that will allow them to develop hypotheses for discovering the identity of their unknown (from a list of potential candidates). Subsequent to the identification of their unknown translation factor, a series of protein unfolding exercises are performed employing circular dichroism and fluorescence spectroscopies, allowing students to directly calculate thermodynamic parameters centered around determining the equilibrium constant for unfolding as a function of denaturant (temperature or chemical). The conclusion of this multi-part laboratory exercise consists of both oral and written presentations, emphasizing synthesis of the roles of each translation factor during the stepwise process of translation.

Keywords: protein folding, biophysical chemistry, translation, circular dichroism, intrinsic tryptophan fluorescence.

Western Washington University is a public predominantly undergraduate institution of approximately 12,000 undergraduate students. The departments of chemistry and biology each has an interdisciplinary major: biochemistry (chemistry department) and cell and molecular biology (biology department). Together, the two majors graduate approximately 40 undergraduates per year. The curricula between departments differ subtly, but both majors require a one-quarter molecular biology laboratory course taught in the biology department, and a one-quarter biochemistry laboratory course that is taught in the chemistry department. The biochemistry laboratory course consists of two 4-hour laboratory meetings and a 1-hour lecture meeting per week for the 10-week quarter. The experiments described herein encompass 6 weeks of the quarter, and are an adaptation of a previously described laboratory module [1–4].

The broad focus of the biochemistry laboratory course is on protein chemistry. For this 6-week module, students begin with a bacterial expression plasmid containing an unknown gene of interest and subsequently complete a series of experiments that express, purify, identify, and analyze an unknown translation factor. Determination of the student's unknown is based on comparison of their experimental results with data for a pre-established list of potential protein candidates (Table I). Each of the unknown translation factors are *Escherichia coli* proteins that are relatively well behaved in the context of the experiments to be completed within this laboratory module, and each is subcloned into an identical expression plasmid for comparable expression and purification protocols. To determine the identity of the unknown protein provided, each student experimentally determines the *E. coli* growth rate doubling times, molar extinction coefficient, molecular weight based on SDS–PAGE, and secondary structure determined from circular dichroism. After the identification of their unknown, the final experiment is to analyze the thermodynamics of protein unfolding for their respective proteins spectroscopically, using at least two methods of protein denaturation (guanidine hydrochloride, urea, temperature, and/or pH) measured by two different techniques (far-UV circular dichroism and intrinsic tryptophan fluorescence). At the conclusion of the experimental section of the laboratory exercise, each student prepares a written report in a peer-reviewed journal format (e.g. Biochemistry), and gives an oral presentation (complete with visual aids, including experimental data and supplemental background information from the primary literature) to the entire class.

The benefits of this experiment in the training and education of undergraduates in biochemistry and molecular

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TABLE I
Translation factor information

Translation factor	MW (kDa)	Theoretical pI	Molar extinction coefficient	Secondary structure	Expression/purification notes
EF-G	77.6	5.24	61,310	α/β	Induction at 15 °C is most efficient
RF1	40.5	5.15	21,430	α/β	Easy, slow cell growth
RF3	59.6	5.65	41,370	α/β	Easy
EF-P	20.6	4.90	25,440	Mostly β	Lower expression levels
RRF	20.6	6.62	2,980	Mostly α	Easy, no tryptophan residues
LepA (EF4)	66.6	5.40	39,310	α/β	Slow growth, small percent of precipitation in dialysis

EF-G, elongation factor G; RF1, release factor 1; RF3, release factor 3; EF-G, elongation factor P; RRF, ribosome recycling factor; EF-4, elongation factor 4 (also called LepA); MW, molecular weight; kDa, kilodaltons; pI, isoelectric point.

biology are several-fold. First, students are exposed to a number of indispensable techniques that are the mainstay for most biomolecular scientists. Second, providing a scenario where unknowns are given to the students has notable value. Students are expected to formulate hypotheses based on their experimental observations, which must be tested with further experiments to correctly identify their unknown. Lastly, there is additional value in the use of multiple unknown proteins within a laboratory section. Students will become aware of the variability of protein solution behavior due to the differing physicochemical properties that the various translation factors possess, including: variable solubility limits, dramatically different molar extinction coefficients, and changes in bacterial growth rates. During the oral presentation section of this experiment module, students are expected to present their identified and analyzed translation factor in the context of the complete biological process, learn about different translation factors from fellow student presenters, and mentally synthesize the entire process of prokaryotic translation.

The procedures to determine unfolding thermodynamics values of each protein provide an accessible means for understanding complex course material that students often struggle to learn [5]. The experiments presented in this laboratory module allow students to calculate the Gibbs free energy of unfolding by two principal methods: chemical and temperature denaturation. Students collect temperature denaturation data to construct a protein unfolding curve that allows direct determination of the melting temperature (T_m), equilibrium constants at different temperatures within the transition region (K_{eq}), ΔH°, ΔS°, and finally ΔG° by using the van't Hoff analysis (described below) [6]. The limits of this procedure (assumption of $\Delta C_p = 0$) are briefly explained in the lecture portion of this course (this is expanded upon in a senior-level biophysical chemistry lecture course) [7]. Protein denaturation by chaotropic agents (guanidine hydrochloride or urea) provides data to construct protein unfolding curves. Determination of the equilibrium constant (K_{eq}) at different denaturant concentrations within the transition region allows for linear extrapolation to the absence of denaturant for determination of $\Delta G^\circ(H_2O)$ (see below) [8]. By getting exposure to a variety of techniques, students can compare and contrast thermodynamic calculations and discuss the origins of differences and/or similarities for each approach.

EXPERIMENTAL OUTLINE

Lab Periods 1–2: Bacterial Transformation, Growth, and Protein Overexpression

During the first laboratory period, students start with a bacterial cell culture or cell pellet that is used for performing a standard plasmid preparation (Qiagen, Valencia, CA). The plasmid used for all of the translation factors is pSV281 (a pET15b derivative, kanamycin resistant). The pSV281 plasmid is an isopropylthio-β-D-galactoside (IPTG)-inducible protein expression plasmid containing an N-terminal tobacco etch virus (TEV) protease-cleavable 6X histidine tag for subsequent protein affinity purification. Subsequent to the purification of their plasmid, students perform a cell transformation of their respective plasmids via standard heat shock protocols. At the stage of plasmid purification, the instructor has the option to use restriction enzyme digests to determine the size of their unknown gene (all translation factors are cloned into the pSV281 plasmid between *Bam*HI and *Xho*I sites for the N- and C-termini, respectively). The second laboratory period encompasses large-scale bacterial cell growth (1 L/unknown protein sample) and preparation of buffers for protein purification. Students follow the doubling times of their respective cell cultures to determine when mid-log phase is reached (A_{600} values measured with Spectronic-20 instruments). Once mid-log phase is reached ($A_{600} = 0.4–0.8$), students induce their cultures by adding IPTG to 0.5 mM and allowing overnight growth at 15 °C or room temperature.

Lab Periods 3–6: Protein Purification, Quantitation, Dialysis, and SDS–PAGE

During the third laboratory period, cell pellets containing over-expressed translation factors are resuspended, lysed by sonication, and clarified by centrifugation. During the centrifugation step, students assemble a chelated nickel affinity column (Ni-NTA, Qiagen) and equilibrate the column for binding of the histidine-tagged translation factors. Students collect 2 mL fractions from the initial binding of the supernatant throughout the entire purification protocol. Here, we typically use low-pressure column assemblies (Bio-Rad, Hercules, CA) and generic peristaltic pumps. The absorbance of each fraction is determined following the purification protocol at 280 nm with a UV/Vis spectrophotometer. The resulting elution profile is analyzed to determine which fractions most likely contain

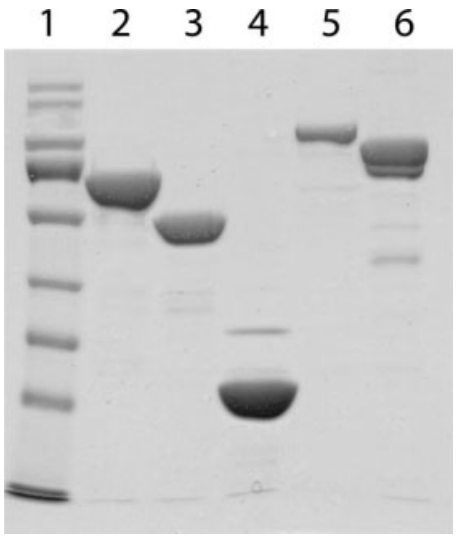

FIG. 1. **SDS–PAGE of one-step affinity purification of translation factors**. Lanes: (1) protein molecular weight standards, (2) RF3, (3) RF1, (4) RRF, (5) EF-G, (6) LepA.

purified translation factor. The fractions hypothesized to contain protein are then quantified by the Bradford assay (Thermo Scientific, Waltham, MA). Students are instructed to develop a standard curve based on known bovine serum albumin concentrations and then quantify their respective protein concentrations by linear regression analysis. Using the calculated molar extinction coefficients for each unknown translation factor (ProtParam, ExPASy proteomics server), students are required to further calculate the mass extinction coefficients for every protein on the list of potential unknowns and compare these values to the experimentally determined mass extinction coefficient for their unknown. Conclusion of the protein purification consists of dialysis of the fractions containing sufficient protein concentrations into the appropriate storage buffer at 4 °C until the next laboratory period. During the fifth laboratory period, students analyze their protein purifications by standard SDS–PAGE methodologies. Here, we prefer a 15% Tris–glycine gel system to provide a broad range of separation to resolve the variety of different molecular weights for the unknown translation factors (Fig. 1). A continuous gel system is sufficient, although a discontinuous SDS–PAGE (separate stacking and resolving regions of the gel) provides better resolution. Samples loaded into the gel typically are: protein molecular weight standards, whole cell lysate, centrifugation supernatant, centrifugation pellet, flow-through, wash fractions, and eluted purified fractions. Upon completion of the SDS–PAGE, the gels are stained in Coomassie blue overnight. During the sixth laboratory period, students destain their SDS–PAGE gels by standard protocols and analyze their resultant bands by calculating the relative migration (R_m) and compare them to the protein molecular weight standards to estimate the molecular weight. Graphical representation of log MW vs. relative migration provides a linear trend to calculate the molecular weight of each protein band in their samples.

Internet Resource Research

Throughout the duration of the entire experiment, students are expected to use the Internet for collecting a variety of data for the translation factors involved in this module (e.g. PubMed, BLAST, Protein Data Bank, and the ExPASy proteomics server). Students are given no more than the name of each translation factor and its origin (*E. coli*). From there, students can access all of the protein sequences and subsequently analyze their properties (sequence conservation, molecular weight, isoelectric point, molar extinction coefficient, predicted secondary structure, and determined tertiary structure). Subsequent to discovery of the identity of their respective unknowns, students are expected to complete a more exhaustive literature search for specific information about the structure and function of their given protein. All of the collected information is incorporated into the oral and written presentations at the end of the laboratory module.

Lab Periods 7–9: Circular Dichroism, Intrinsic Fluorescence, and Thermodynamics

During the seventh laboratory period, students are introduced to the instrumentation for their protein denaturation experiments (Olis, Bogart, GA) CD spectropolarimeter and PTI (Birmingham, NJ) fluorescence spectrophotometer) while they collect initial spectra of their protein in its native state and in a completely denatured state (>75 °C and >6 M urea or guanidine hydrochloride). The students assess this initial data and experimental conditions to refine their experimental conditions for more comprehensive protein denaturation experiments that are performed during the eighth and ninth laboratory periods. Typically, students will use one spectroscopic technique to perform temperature denaturation and the other spectroscopic technique to measure chemical denaturation. At this step, the students are to develop their own protocol for how each particular experiment is to be performed, in consultation with an instructor. This allows the student to step away from following recipe-type laboratory procedures and think independently about crucial experimental factors, such as protein concentration, temperature steps, incubation times, titration volumes, etc. Using their denaturation data, students employ a variety of calculations to determine various thermodynamic parameters.

During chemical denaturation, the instructor typically must encourage the students to consider such factors as protein, buffer, and denaturant concentrations for the duration of the titration. We suggest a two solution approach, where one formulates a “native” solution of protein that is absent of denaturant, and a “denatured” solution that contains the protein under the same concentration and buffer formulation with the addition of enough denaturant to render the protein completely unfolded (>4 M guanidine hydrochloride, >7 M urea). Once these two solutions are made, students can remove defined amounts of “native” protein and subsequently add defined amounts of “denatured” protein in a step-wise manner that allows for control of all other variables (for a 3 mL titration, step-wise exchange of 200 μL is sufficient). This type of titration additionally provides the option of performing the reverse titration if desired.

For thermal denaturation protocols, we utilize a cuvette that is directly connected to a Peltier device. For brevity, we typically adjust the temperature in 2–3 °C intervals with 2-minute incubation times between readings. An in-line Peltier device additionally allows measuring thermal reversibility. One can perform a temperature denaturation with a series of incubators at different temperatures, however, results are more laborious and less accurate.

During the CD experiment, proteins have their initial spectra scanned in the far-UV region between 205 and 265 nm. This measures the type and amount of protein secondary structure, which may aid in determining the identity of the unknown protein [9]. Once a clear spectrum has been collected for each student's protein, they select a wavelength that is indicative of their folded protein (usually 216–222 nm) and collect spectra in this region during the titration of denaturant.

Intrinsic tryptophan fluorescence is performed with an excitation wavelength of 280 nm and emission wavelength spectra between 320 and 360 nm. During the titration of denaturant, complete fluorescence spectra are collected to directly monitor intensity changes and peak wavelength shifts [10]. Upon completion of the protein denaturation experiments, data are appropriately plotted for calculation of thermodynamic parameters by different methods.

Thermodynamic calculations from thermal denaturation data consist of using a van't Hoff analysis [6]. Here, students are to plot the thermal denaturation data, separate their data into three different regions (pre- and post-transition, and transition regions), and perform a linear regression analysis for each region independently (Fig. 2*A*). Provided that simple thermodynamic assumptions are made (reversibility, two-state process $N \leftrightarrow D$, $\Delta C_p = 0$), students can accurately calculate changes in the fraction of unfolded protein (α) and correspondingly, the equilibrium constant (K_{eq}) as a function of temperature in the transition region. Here, one can start with spectroscopic measurements in the transition region and combine this value with the extrapolated values for the pre- and post-transition baselines to calculate α (equation listed below). Subsequent graphical analysis is then used to generate a van't Hoff plot (ln K_{eq} vs. 1/T) (Fig. 2*B*). The slope of this plot determines the enthalpy of unfolding (ΔH°), and the y-intercept is related to the change in entropy during unfolding (ΔS°). Finally, the Gibbs free energy of unfolding is calculated from the entropic and enthalpic contributions at a given temperature ($\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$). Using two different spectroscopic techniques, students can compare their data for a given translation factor to investigate what temperature secondary structure (CD) denatures in contrast to tertiary structure (fluorescence) denaturation. The equations below describe the determination of α, K_{eq}, and the van't Hoff relationship (ln K_{eq} vs. 1/T).

$$\alpha = \frac{A_{obs} - A_N}{A_D - A_N},$$

$$K_{eq} = \frac{[D]}{[N]} = \frac{\alpha}{1-\alpha} = \frac{A_N - A_{obs}}{A_{obs} - A_D},$$

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ = -RT \ln K_{eq},$$

$$\ln K_{eq} = \left(\frac{1}{T}\right)\frac{-\Delta H^\circ}{R} + \frac{\Delta S^\circ}{R}.$$

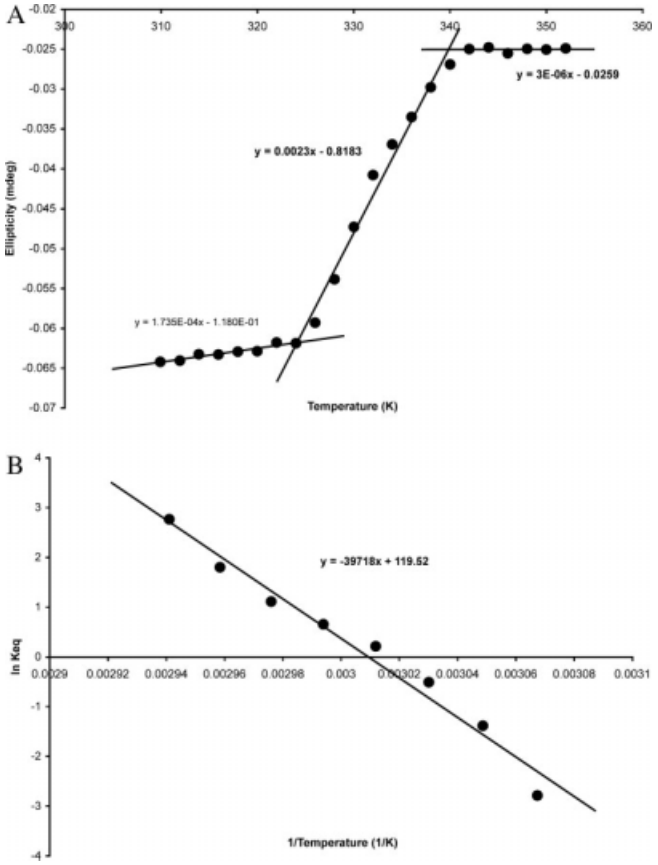

FIG. 2. **Thermal denaturation of EF-G**. (*A*) Circular dichroism at 220 nm as a function of temperature. Each region of the graph is fit by linear regression (pre-transition, post-transition, and transition regions). (*B*) van't Hoff plot resulting from the circular dichroism spectra as a function of temperature. The y-intercept represents $\Delta S^\circ/R$, and the slope represents $-\Delta H^\circ/R$.

Chemical denaturation by the addition of chaotropic agents (urea and guanidine hydrochloride) is a common method used for determining the Gibbs free energy of unfolding [8]. Here, one can monitor the fraction of unfolded protein as before (α) as a linear function of denaturant concentration within the transition region and plot the Gibbs free energy (as derived from K_{eq}) (Fig. 3*A*). Once this is done for the narrow denaturant concentration range in the transition region, one can extrapolate to the y-axis to determine the Gibbs free energy of unfolding in the absence of denaturant, $\Delta G^\circ(H_2O)$ (Fig. 3*B*). As before, students monitor the three regions of the unfolding curve to establish the pre- and post-transition baselines for accurate determination of K_{eq} in the transition region. The significance of the transition region slope (m_g) is not discussed in detail, as it is a subject of the required biophysical chemistry lecture course for biochemistry majors [7]. Given a variety of different denaturants and spectroscopic techniques, students can compare the effects of urea versus guanidine hydrochloride as denaturing agents, and compare the hierarchy of folding as described above for thermal denaturation. The equations listed below indicate the relationship between $\Delta G^\circ_{(obs)}$ and $K_{eq,obs}$ at a given denaturant concentration

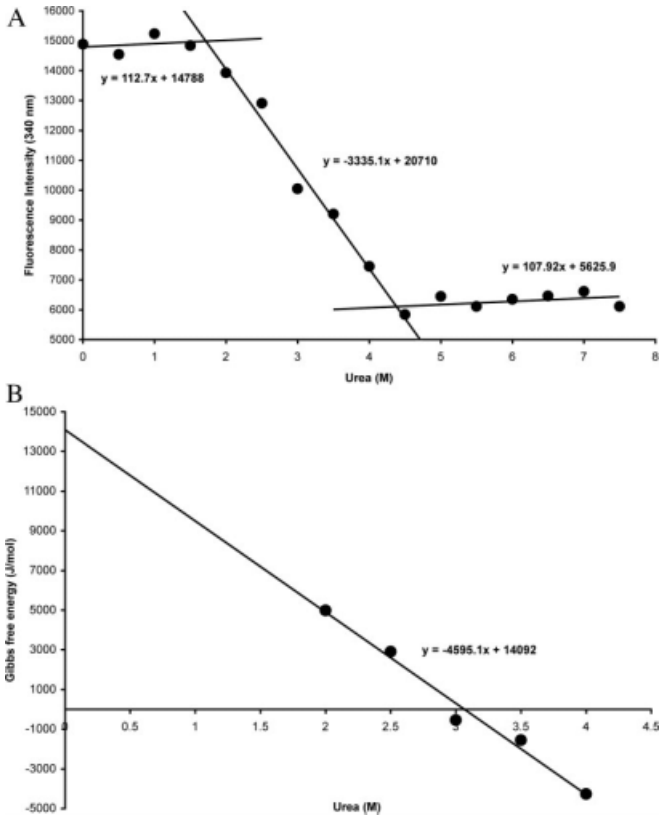

FIG. 3. **Chemical denaturation of RF3**. (*A*) Intrinsic tryptophan fluorescence (excitation: 280 nm, emission: 340 nm) as a function of urea concentration. Each region of the graph is fit by linear regression (pre-transition, post-transition, and transition regions). (*B*) Linear extrapolation from ΔG°(obs) at different urea concentrations. Extrapolation to the *y*-axis allows determination of $\Delta G^{\circ}(H_2O)$.

and shows the relationship between the $\Delta G^{\circ}_{(obs)}$ and $\Delta G^{\circ}(H_2O)$ (in the absence of denaturant).

$$\Delta G^{\circ}_{obs} = -RT \ln K_{eq,obs},$$

$$\Delta G^{\circ}_{obs} = \Delta G^{\circ}_{H_2O} + m_g[\text{denaturant}].$$

Written and Oral Presentation

As it is organized at WWU, this course fulfills a portion of a writing proficiency requirement. Therefore, it is inherently writing intensive. Before each section of the laboratory module, students are required to construct a prelaboratory report in a peer-reviewed journal format. The prelaboratory consists of a brief abstract, introduction, and methods. Upon completion of each section, the students are also required to write a postlaboratory, which includes a results and discussion section. The content for the pre- and postlaboratories are mostly experimental in nature, with information about the methods performed, the theoretical background and utility of each method, and a brief description of the project. Upon completion of the laboratory module, the students synthesize a final laboratory report that contains much of the information already written in the pre- and postlaboratories including editorial suggestions from the instructor, as well as a significant amount of background for their particular translation factor that is bolstered by the primary literature. Information must be incorporated into the laboratory report that includes the structure and function of each translation factor, which is properly cited from peer-reviewed literature. In addition to the laboratory report, each student (or pair) prepares a 20-minute oral presentation that is given to the entire class. These presentations catalyze interactive discussions about each project during the question and answer section of each talk. Here, students directly learn about each translation factor in far greater detail than is typically accomplished in a large lecture course.

SUMMARY

Upon completion of this set of laboratory exercises, students gain valuable exposure to many fundamental techniques in biochemistry, learn to analyze thermodynamic processes spectroscopically, and learn how to formulate hypotheses from experimental data. During the first six laboratory periods, students grow familiar with the processes that entail generating and analyzing protein reagents as needed for further biochemical analysis. This is an important realization for undergraduates, as preparation of biochemical reagents can be significantly different than for most other chemistry instructional labs. With each successive experimental procedure, students discover further information about their unknown translation factor. This serves as a means to keep students interested in their laboratory experience, it illustrates the inherent limitations of data collected by a variety of fundamental techniques, and allows students to derive their own hypotheses and conclusions that are unique to their particular protein.

Following the initial characterization and identification of each unknown translation factor, students examine their purified proteins with a more complex thermodynamic analysis of their particular protein. During this section, students are assigned different denaturant methods for thermodynamic analysis. Upon completion of the denaturation data collection, students directly assess their respective data to calculate thermodynamic parameters based on the procedures described above, which are explained in the lecture portion of the class. This step is an obvious roadblock for many students, as thermodynamics is often inaccessible in the context of a lecture course [5].

While the experimental procedures and contiguous laboratory format are both valuable and well documented as an effective teaching tool [3, 4], the unique strength of this particular laboratory module arises from the incorporation of a suite of unknowns that are all connected through a fundamental biological process. First, the ability to start with an unknown protein, perform initial experiments to allow the formulation of rough hypotheses, and then having the opportunity to test and validate these hypotheses with further experimental procedures is an important skill to develop at the undergraduate level. Secondly, by examining a series of proteins that are connected biologically to each other, students can bridge a connection between the individual proteins that are being examined with an entire biological process. This is dra-

matically apparent during the oral presentation section of this laboratory module. Here, students openly discuss the role(s) of each translation factor, and synthesize the order and importance of each step in the prokaryotic translation cycle.

By implementing several different proteins in an instructional laboratory environment, the procedures and heterogeneity for each process can become cumbersome. In selecting prokaryotic translation factors for this experiment, one can minimize this complexity. Each of the translation factors expresses and purifies well by one-step affinity purification. One must also consider the number of unique properties that are specific for different factors. For instance, RRF contains no tryptophan residues, and thus cannot be analyzed effectively in the fluorescence experiment. LepA and RF1 cause cells to grow slower, including growth before induction with IPTG. To aid in the literature research portion of this module, students are given proteins that have been comprehensively described in the scientific literature, for which three-dimensional structural data is available. It is expected that a portion of the written and oral presentations will consist of sufficient structural and functional background for their particular translation factor that describes their protein at a level deeper than what is traditionally found in a Biochemistry textbook.

Student assessment regarding the content and format of this laboratory module has been positive. Retrospective comments from students included: "The entire process gave me a glimpse of what goes into basic science research, as well as the skills one needs to have to be a successful scientist," "Trying to identify an unknown made the laboratory more interesting. Having a list of possibilities made it manageable, but still gave students a reason for investment in the project," "Each protein presented its own challenges in purification and characterization like those encountered in the research field. I felt this made the experience less like the typical 'working from a cookbook' lab," and "Oral presentations on each of the translation factors provided students with a strong overview of translation."

FUTURE CONSIDERATIONS

Currently, this experiment only uses a subset of the translation factors that are central to the process of prokaryotic translation (EF-G, RF1, RF3, LepA, RRF, and EF-P). To complete the entire translation process, we have recently cloned IF1, IF3, and EF-Tu. Future iterations of this module might consist of scheduling the student oral presentations to coincide with the order of steps in translation (initiation lectures first, recycling lectures last, etc.) As this module develops further, we propose to incorporate additional techniques, such as ESI-MS for molecular weight determination, GTPase activity assays for the various GTPases as a measure of enzyme activity, ribosome-binding experiments, and TEV protease cleavage assays. At WWU, the remaining laboratory exercises during the last 4 weeks of the quarter consist of protein molecular weight determination by electrospray ionization mass spectronetry (ESI-MS), preparation and Western blot analysis of myoglobin, and a steady-state enzyme kinetics study of acetylcholine esterase.

The laboratory manual, detailed instructions, and plasmid reagents are available upon request to the corresponding author.

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