STRUCTURE LEADS TO FUNCTION: AN INTEGRATED BIOPHYSICAL APPROACH TO TEACHING A BIOCHEMISTRY LABORATORY

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We have developed and implemented an integrated approach to teaching our biochemistry laboratory. The central theme of our two semester course focuses on the relationship between the three-dimensional structure of a macromolecule and its function. While the first semester lecture focuses in part on the structure and function of protein enzymes, we have found it convenient in the second semester laboratory to focus on the three-dimensional structure of RNA. We chose RNA as our model system for two reasons. First, there are many examples of RNA enzymes in the literature and they fold into unique and complex tertiary structures to function. Second, unlike proteins, small RNA molecules are easily synthesized enzymatically in the laboratory and the thermodynamic parameters can be determined by relatively simple methodologies.

Central to the development of our laboratory was the construction of a curriculum designed to approach research similar to how science is done in the real world. Moreover, a multidisciplinary approach was taken to introduce many different biochemical techniques to the study of a single biophysical system. Students determined the thermodynamic parameters of two related RNA hairpins. Then investigations include designing the single-stranded T7 templates, purifying the DNA oligonucleotide templates, transcribing and purifying the RNA hairpins, quantifying DNA and RNA oligonucleotides, performing thermal melting studies on the UV-vis spectrophotometer, and analyzing data to determine the relevant thermodynamic parameters of the RNA model system.

Background and Course Goals
RNA is involved in a variety of biological functions. Several RNAs are required for protein translation; rRNAs, tRNAs, and mRNAs (Trachsel, 1991). Recently the Escherichia coli 23S rRNA was implicated in the peptidyl transferase activity during translation (Noller et al., 1992). Formation of the spliceosome and removal of the intron from pre-mRNAs requires a combination of five different snRNAs and splicing factors (Nilson, 1994). Numerous catalytic RNAs have now been characterized that function on a variety of different substrates (Gesteland and Atkins, 1993).

The functions of these various RNAs depend on their abilities to form three-dimensional structures (Chastain and Tinoco, 1991). An RNA’s three-dimensional structure is composed of a primary sequence of nucleotides which interact to form secondary structural elements which, in turn, interact to form a tertiary structure. The tertiary interactions that promote formation of the three-dimensional structure are generally assumed to be weaker than the sum of interactions that form the secondary structures (Turner et al., 1988). Thus, the total free energy of formation for a three-dimensional RNA can be approximated by the sum of free energies of formation for its secondary structures.

Secondary structure is formed by the matching of palindromic sequences within the primary sequence, usually purine-pyrimidene base pairs, though other, non-Watson-Crick base pairs have been noted (Saenger, 1984; Morse and Draper, 1995). Secondary structural elements can include double helices, external loops, internal loops, and bulges (Chastain and Tinoco, 1991; Tinoco et al., 1987).

There are several approaches to predicting RNA secondary structure, including phylogenetic studies, structure mapping, and thermodynamic stability (Serra et al., 1994; Turner et al., 1988). The best approach for defining RNA secondary structure is by using a combination of all three
methodologies. The main limitation of the thermodynamic approach is the lack of data for secondary elements other than helices and small external loop sequences (Serra et al., 1994).

External loop stability is dependent on the size of the loop, but may not be dependent on its composition (Groebe and Uhlenbeck, 1988). The stability of an external loop is also dependent on the closing base pair of the loop and the first mismatch in the loop (Serra et al., 1993; Serra et al., 1994). Bulge nucleotides have also been found to affect hairpin stability (Groebe and Uhlenbeck, 1988).

In contrast to external loops in RNA secondary structure, much less is currently known about internal loops and their contribution to overall thermodynamic stability of secondary structure motifs. Most notably, Santa Lucia et al. (1991) have shown that an internal loop stabilizes the duplex when the mismatches are G•A, U•U, or C•C+, and destabilizes the duplex when the mismatches are G•G, C•A, C•U, A•A, or C•C. Whether these trends exist in the internal loops of biologically important RNAs has been the subject of much speculation; therefore, we adopted this question as the working hypothesis for the laboratory. The goal of our course was to determine the thermodynamic contribution of the internal loop to the overall stability of a naturally occurring RNA hairpin (Figure 1). By using in vitro transcription the students synthesized two small RNA hairpins (Figure 1b and 1c), determined their thermodynamic stability, and calculated the thermodynamic contribution of the internal loop to the overall stability of the naturally occurring RNA hairpin.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{(a) Portion of the naturally occurring C. elegans U6 spliceosomal snRNA (Thomas et al., 1990). (b) U61 Hairpin. (c) U62 RNA hairpin minus the internal loop.}
\end{figure}

RESULTS

Course Outline and Structure
Our biochemistry laboratory consists of one three hour laboratory per week. The work described in this manuscript requires considerably more time and it was common for the students to spend about 6-8 hours per week in the laboratory. The class consisted of 18 students that were divided into six groups of three for the duration of the semester. From a teaching perspective, the course was divided into four main parts including macromolecular structure and function, preparation of transcription templates, RNA synthesis, and UV melting studies of the RNA hairpins.

Macromolecular Structure and Function
This portion of the laboratory lasted about four weeks and consisted of lectures and discussions in order to prepare the students for the rigors of the project. During the first laboratory session each group was handed a packet consisting of the relevant literature needed to complete the project. The tutorials started by focusing on the theoretical aspects of proteins and nucleic acids as important biological molecules and finished off emphasizing RNA and the importance of three-dimensional structure to its function. The last two weeks were devoted to the study of the thermodynamics of RNA structure and theoretical aspects behind the biochemical and biophysical techniques used to obtain these quantities.

Preparation of Transcription Templates

A. Purification of the single-stranded DNA templates

In order to carry out in vitro RNA synthesis using the Milligan method (Milligan et al., 1987), the students must first purify and quantitate the top and bottom strands of the transcription tem-
plate (Figure 2). DNA oligonucleotides can be conveniently ordered from a number of different companies thereby obviating the need for the chemical synthesis of the template strands on-site. For a class of 18 we ordered a 1 μM synthesis of each of the template oligonucleotides which was more than enough DNA for the laboratory. Choice of the template sequence is critical and we have relied on the conditions to be published elsewhere (deLannoy et al., 1996). In this case, template oligonucleotides were obtained from Oligos Etc., the lyophilized powder adjusted to 500 μl with deionized water, and aliquoted into 40 μl samples ready for student use. Each group was presented with DNA correlating to the bottom strand of the U61 and U62 templates. The top strand was purified ahead of time by the instructor. Each group combined 20 μl of formamide (Sigma Chemical Company) with their samples and fractionated the mixture on a 20% polyacrylamide-7 M urea gel in 89 mM Tris-Boric Acid, pH 8, 2 mM EDTA (20 cm gel and 1 mm spacers; Maniatis et al., 1982). The relevant bands were identified by UV shadowing, cut out, and eluted overnight at 4°C in 10 ml of deionized water. Prior to the addition of the water, the gel slice was crushed in a 14 ml- 17x100 mm polypropylene centrifuge tube. Following overnight elution, the supernatant was transferred to a clean dry polypropylene tube and evaporated to 500 μl using the rotary speed-vac evaporator. The students were allowed one week for the evaporation step due to a time requirement of 4-6 hours and they were requested to come into the laboratory on their own time to complete this step. However, depending on local contexts, the instructor may want to do this step for the students. The purified oligonucleotides were precipitated with two volumes of cold ethanol and 1/10 volume 3 M sodium acetate and stored at -20°C. The procedures noted above and those described below should be completed wearing gloves and using sterile solutions. We have found that small structured RNAs are less susceptible to contaminating nucleases than large RNAs; nonetheless, care should be taken when handling the template oligonucleotides as well as other relevant experimental manipulations described below.

B. Quantification of the single-stranded DNA templates

The resulting oligonucleotides were quantified by UV-vis spectroscopy using extinction coefficients described previously (Puglisi and Tinoco, 1989). The resulting pellets from above were brought up in 50 μl of distilled water, a 1 μl aliquot diluted 500-fold, and the absorbance measured at 260 nm. For U61 DNA, usually three gel preparations were combined as one isolation procedure which provides a DNA stock solution of approximately 80-120 μM (one gel preparation was 40 μl of crude DNA). The T7 top strand oligonucleotide should be in the range of about 300 μM. Typically the yields were better for the small oligonucleotides than for the large ones.

RNA Synthesis

A. Hybridization of the template strands

The goal of this portion of the laboratory was to generate the templates needed for T7 transcription of U61 and U62 RNAs (Figure 2a and 2b). In this step equal amounts (350 pmols) of the top and bottom strands of the transcription template were combined in 10 μl of appropriate buffer (10 mM Tris, pH 7.5, 10 mM NaCl, and 0.1 mM EDTA), heated to 100°C for two minutes and snap cooled on ice. The templates must be prepared just prior to performing the transcription since storage of the templates at -20°C decreases the efficiency of transcription (deLannoy et al., 1996).

B. T7 RNA Synthesis

RNA hairpins were synthesized by the in vitro transcription of single-stranded DNA templates by T7 RNA polymerase (Milligan et al., 1987; Ambion, Inc.). Unless otherwise stated the conditions described below were standard procedures from Ambion Inc., with the following modifications. A standard overnight transcription contained the appropriate template (350 pmols; 10 μl from above), and 400 U of additional T7 RNA polymerase in a total volume of 100 μl. Usually, three transcription reactions were set up side by side for a given template, incubated overnight at 37°C and stopped by the addition of 10 U of RNase free DNase I. The volume of each reaction was adjusted to 200 μl with deionized water and extracted one time with 100 μl of chloroform/isoamyl alcohol (24:1) and precipitated with three volumes of cold 95% ethanol. The transcription mixtures were subsequently fractionated on a 20% polyacrylamide-7 M urea denaturing gel as described above. The appropriate band was identified by UV shadowing, purified and quantitated by the procedures outlined above (Maniatis et al., 1982). For transcripts, usually three gel lanes were combined as one isolation procedure which provides an RNA stock solution of approximately 300 μM in 50 μl of deionized water (one transcription preparation was loaded in one gel lane).
Figure 2. The single-stranded DNA templates used for the in vitro transcription of the (a) U61 and (b) U62 RNA hairpins are shown above.

**UV Melting Studies of RNA Hairpins**

**A. RNA Hairpin Sample Preparation**

The conditions used for the melting studies were by Puglisi and Tinoco, (1989) with the following modifications. The buffer used for thermodynamic studies was 10 mM sodium cacodylate, pH 7 with NaCl as specified below. In these experiments, NaCl concentration was maintained at 10 mM with an RNA concentration of 3 μM. The specific amounts needed of each were determined based on the molarity of the stock solutions and the fact that total volume required for the quartz cuvette (0.5 cm path length, Hellma Cells, Inc.) was 200 μl. Samples were mixed taking care to centrifuge between each additional aliquot. When all aliquots had been combined in the Eppendorf tubes, the RNA sample was placed in a water bath, brought to a rolling boil for 3 minutes, and snap cooled in ice. The sample was again centrifuged and loaded in the cuvette for spectral measurements. After the sample was loaded, the cuvette was placed into the cell holder.
C. Data Analysis

Upon completion of each experiment, the results were checked graphically on the UV-vis spectrophotometer. The typical melting curve which was obtained is shown below in Figure 3.

The acquired data was transferred from the UV-vis spectrophotometer to a Power Macintosh 6100/66 where they were imported into a spreadsheet package (Cricket-Graph III, version 1.5.3) and converted to vector format. After the transformation, the data in vector form were transferred into Student Edition of Matlab 4a for Macintosh (The MathWorks, Inc.). This program is a numeric computational tool which allows the determination of the thermodynamic parameters of the hairpins based on the van't Hoff relation and standard thermodynamic equations described previously (Puglisi and Tinoco, 1989). Once the thermodynamic parameters were obtained, the standard deviations for the experiments were determined. The data were accepted only if the standard deviations were within published experimental error values (Santa Lucia et al., 1991). The thermodynamic parameters obtained are noted in Table 1.

Discussion

The laboratory described above was implemented in the spring semester of biochemistry at Black Hills State University. The course was an ambitious undertaking and required a large time commitment from both the students and the faculty member who taught the course. All or part of the laboratory described may be adopted or used by others. For example, a shorter more abbreviated laboratory regarding the thermodynamics of RNA structure could be taught by simply providing the students with the appropriate RNA for melting analysis. A laboratory of this nature could conveniently be completed over a four week period including the time required for theoretical discussions.

Table 1. Thermodynamic parameters of 3 μM U61 and U62 at 10 mM NaCl. The Tm varied +/- 0.19°C and the standard deviation was ±4.9 kcal/mol for ΔS°, ±15 cal/(mol-deg) for ΔH° and ±0.14 kcal/mol for ΔG°. The data shown below were determined from a minimum of two melting profiles each.

<table>
<thead>
<tr>
<th>Tm (°C)</th>
<th>ΔS° (cal/mol-deg)</th>
<th>ΔH° (kcal/mol)</th>
<th>ΔG° (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U61</td>
<td>45.16</td>
<td>-182.2</td>
<td>-57.9</td>
</tr>
<tr>
<td>U62</td>
<td>62.44</td>
<td>-222.3</td>
<td>-74.6</td>
</tr>
</tbody>
</table>
Additionally, other templates could be chosen in order to synthesize and analyze other small RNA structures (Milligan et al., 1987). However, we recommend that other potential sequences maintain the first two Cs in the template since removing them resulted in significantly decreased transcription yields as previously reported (Milligan et al., 1987). Moreover, care must be taken to set up the transcription reactions at room temperature since the transcription buffers used for these reactions contain spermidine which would precipitate the DNA templates if the reactions were set up on ice. Finally, one may also choose not to use the transcription kits from Ambion, Inc.; however, we have found them easy to use, have optimized the templates described above using this system, and the kits can be stored at -20°C for at least one year with little or no loss in T7 RNA polymerase activity.

Denaturing gel electrophoresis was used to purify both the DNA oligos and RNA products. Since polyacrylamide is a neurotoxin, it was recommended that the students wear gloves when handling the gels or gel products. Additionally, in order to avoid degradation problems with regards to the RNA transcripts the students were encouraged to wear gloves during the laboratory steps mentioned above and sterile technique was emphasized throughout the laboratory.

Once the RNA was in hand the students were able to complete two melting profiles each day. The students were encouraged to analyze their melting curves immediately following a melt in order to determine the viability of the data. Thus, a new melt could be set up with a fresh sample of RNA if the previous melt failed or the data was statistically invalid. This was important since each melt required about 8 hours to complete and each group was required to obtain two statistically significant melts for U61 and U62 respectively. The data from each group was combined and used by the entire class to complete the group papers and presentations.

In terms of student assessment, each group was required to write a scientific paper and present a seminar describing their results. Initially the students were assigned the abstract and introduction which they had to complete within the first two weeks of the laboratory. This provided the students with the opportunity to obtain a solid grasp of the concepts behind the project and also provided the instructor with a mechanism to determine which groups had conceptual problems. The written portion of the laboratory developed along with the laboratory so that by the end of the semester each group had created a quality product. This resulted in the students approaching science in a real world manner and provided an environment that fostered the mentor-student relationship similar to academic and industrial research laboratories.

Acknowledgments
This work was funded by a grant from the Black Hills State University Faculty Research Fund. We would also like to thank Joseph Howell and Steve Sachs for technical assistance and Dorothy Keller for administrative assistance.

Literature Cited


The American Society for Microbiology recently announced a national curriculum development program for teachers to develop innovative teaching approaches using the "Biofilms" CD-ROM (see page 12). Curricular approaches being sought include both classroom (lecture) and laboratory activities. Application deadline is February 15, 1997. "The CD-ROM fills an important gap where faculty rarely have had copyright free material for classroom use. The seed grants for faculty training are essential because faculty have little experience in using this new technology," says Jean Douthwright of Rochester Institute of Technology and Chairperson of ASM's Division on Microbiology Education. For more information contact:

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ASM/NGMS Undergraduate Research Fellowship: Encourages undergraduate students to conduct a research project in the laboratories of ASM sponsoring members for a minimum of eight weeks. Web Site Address: http://www.asmusa.org/educrsrc/edu23b.htm

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