Characterization of Tertiary Folding of RNA by Circular Dichroism and Urea

Since X-ray crystallography of tRNA was first performed in the 1970s, the study of RNA folding has extended into the characterization of tertiary organization. Although circular dichroism (CD) spectroscopy cannot yet provide structural detail about tertiary organization, it can be used to monitor RNA tertiary folding transitions, which may not be observable by absorbance spectroscopy (Pan and Sosnick, 1997). With the use of computer-controlled titrators, data can be acquired rapidly and accurate thermodynamic parameters can be obtained over a wide variety of conditions. Hence, CD provides a nice complement to site-resolved methods such as complementary oligonucleotide hybridization (Zarrinkar and Williamson, 1994), hydroxyl radical footprinting (Sclavi et al., 1997), or chemical modification (Banerjee and Turner, 1995). This unit provides a basic outline for using CD and urea to measure and characterize tertiary RNA folding transitions to extract thermodynamic parameters.

MEASUREMENT OF A CIRCULAR DICHROISM (CD) SPECTRUM

BASIC PROTOCOL 1

The CD spectrum provides information about the amount of secondary and tertiary structure in the RNA. The changes in the spectrum can be used to identify folding transitions and determine their thermodynamic parameters. Some of these transitions, in particular tertiary transitions, may be unobservable by more standard absorbance measurements.

Materials

Unfolded RNA sample
UV-VIS spectrophotometer
1-cm-path-length quartz cuvette
90°C water bath
Circular dichroism (CD) spectrometer capable of far-UV measurements (e.g., Jasco or AVIV Associates)
Magnetic stir bar to fit cuvette

Additional reagents and equipment for RNA renaturation (*UNIT 6.3*)

Prepare sample

1. Prepare an unfolded RNA sample with absorbance at 260 nm (A_{260}) of ~0.3 to 0.8 AU for optimal signal-to-noise ratio.

   This corresponds to ~10 to 20 μg/mL when using a 1-cm-path-length cuvette (1.5 mL). Increase concentration accordingly for shorter path lengths. The same cuvette can be used for the UV-VIS and CD spectrophotometers.

   Decreasing the RNA concentration will extend measurements further into the UV, but this also decreases the signal. Increasing the concentration will increase the signal, but this decreases the amount of light passing through the sample, and hence lowers the signal quality.

   Keep buffer and chloride concentrations to a minimum as these reagents absorb in the far-UV regions.

2. Perform a renaturing step (*UNIT 6.3*) involving heating to 90°C for 3 min, to remove residual structure formed during purification steps.

Contributed by Tobin R. Sosnick
Current Protocols in Nucleic Acid Chemistry (2001) 11.5.1-11.5.10

Measure CD and absorbance spectra

3. Set up the CD spectrometer using acquisition parameters approximately as follows:

   a. Set resolution or bandwidth at 2 nm.

      All features in the RNA spectrum are broad and resolution need not be higher than this. Decreasing the resolution increases the amount of light passing through the sample and improves signal quality.

   b. Set time constant or response time (time period over which the signal is averaged for each data point) according to the manufacturer's recommendation.

      Generally, this period should be small enough that the CD signal from wavelengths differing by an amount greater than the resolution (e.g., 2 nm) is not averaged in. Shorter time constants will result in decreased averaging and lower signal quality. For example, a scan rate of 20 nm/min is well matched to a response time of 4 sec at 2 nm resolution.

4. Scan from 200 to 320 nm.

   This is the spectral range which contains the RNA structural information. The range from 250 to 320 nm, however, generally contains enough information to characterize the relevant folding transitions. The wavelength scan speed (e.g. 20 nm/min) should be slow, to obtain good signal quality. If the quality is low, multiple scans can be averaged together.

   For a 20 μg/mL RNA sample with an A_{260} of ~0.5 in a 1-cm-path-length cuvette, a 100-nm scan at 2 nm resolution with reasonable signal quality can be taken in ~5 min using an instrumental time constant of 4 sec.

5. Record CD and absorbance simultaneously.

   Most commercially available CD spectrometers simultaneously record CD and the amplitude of the applied photomultiplier voltage (high tension). The latter can be converted to absorbance, enabling one to measure transitions by both CD and absorbance without any additional measurements. See the manufacturer's operational manual for the procedure for converting from high tension to absorbance.

6. Put CD spectrum on an absolute scale normalized to the number of nucleotides, in order that proper comparisons to other spectrum can be made, using the formula for the molar circular dichroic absorption ($\Delta\varepsilon$ in $cm^2/mmol$):

$$\Delta\varepsilon = \frac{\theta}{32{,}980 \times C \times L \times N}$$

Equation 11.5.1

   where θ is the measured (raw) CD amplitude in mdeg, C is the sample concentration in mol/L, L is the cell path length in cm, and N is the number of nucleotides of the RNA.

BASIC PROTOCOL 2

MEASUREMENT AND ANALYSIS OF Mg^{2+}-INDUCED FOLDING TRANSITIONS

The tertiary folding of many RNAs is intimately coupled to the binding of divalent cations, typically Mg^{2+} (Fang, et al., 1999). After the 90°C renaturing step conducted in the absence of cations, the initial state of the RNA may contain some residual secondary structures. Upon addition of metal, further secondary structure as well tertiary structure will form. Near-UV circular dichroism can be used to follow the Mg^{2+} dependence of the folding transitions. The protocol is performed using two titrations. In the first, a coarse Mg^{2+} titration is used to determine which and how many wavelengths are appropriate for monitoring each folding transition. A second, finely spaced Mg^{2+} titration is then performed. Thermodynamic parameters of each transition are obtained from an analysis of the changes in signal as a function of Mg^{2+} concentration. The data from the second

titration are fit to a binding equation to obtain two parameters, the dissociation constant, K_d, and Hill coefficient, n. The Mg^{2+} transition midpoint will be written as K_D (units of molarity) in this unit and is related to the dissociation constant by $(K_D)^n = K_d$ (Fig. 11.5.1).

Materials

Unfolded RNA sample
10 mM and 1 M magnesium stock solutions (ultrapure, autoclaved)
Plastic capillary tubing
Calibrated gas-tight glass syringe
Magnetic stir bar to fit cuvette
Additional reagents and equipment for determining CD spectrum (see Basic Protocol 1)

1. Perform an initial coarse Mg^{2+}-dependence series starting from the unfolded, Mg^{2+}-free RNA followed by a series of spectra at increasing Mg^{2+} concentrations. Add small volumes (e.g., 2 to 20 µL) of a concentrated Mg^{2+} solution to a single sample and correct for the resulting dilution. To avoid excessive dilution, add stock solution of 10 mM $MgCl_2$ until the RNA sample is near 100 mM Mg^{2+}, at which point the 1 M $MgCl_2$ solution should be used to increase Mg^{2+} concentration. Monitor absorbance and CD (see Basic Protocol 1) at each concentration point.

   Generally, an unfolded to intermediate transition occurs at micromolar Mg^{2+} concentrations and is readily monitored by changes in absorbance (A_{260}) and CD at 260 nm ($\Delta\varepsilon_{260}$). These signals are primarily sensitive to the formation of helical structure. Subsequent tertiary transitions generally occur in the millimolar Mg^{2+} range. These may only be measurable by changes in CD.

2. Identify number of structural transitions and choose wavelengths to monitor each transition based upon its particular spectral changes.

   An increase in the CD signal and a decrease in the absorbance near 260 nm are primarily due to base stacking accompanying helix formation. Changes in the CD signal in the region from 275 to 290 nm are useful to monitor tertiary folding transitions. The magnitude of the CD changes depends upon wavelength and can be in the range of 0.1 to 3 cm^2/mmol. The magnitude of the absorbance changes can be 0.01—0.3 × 10^6 M^{-1} cm^{-1}. Often the changes are <10% of the initial CD or absorbance signal. Hence, high-quality data and accurate dilution corrections are a requisite for proper analysis of the folding transitions.

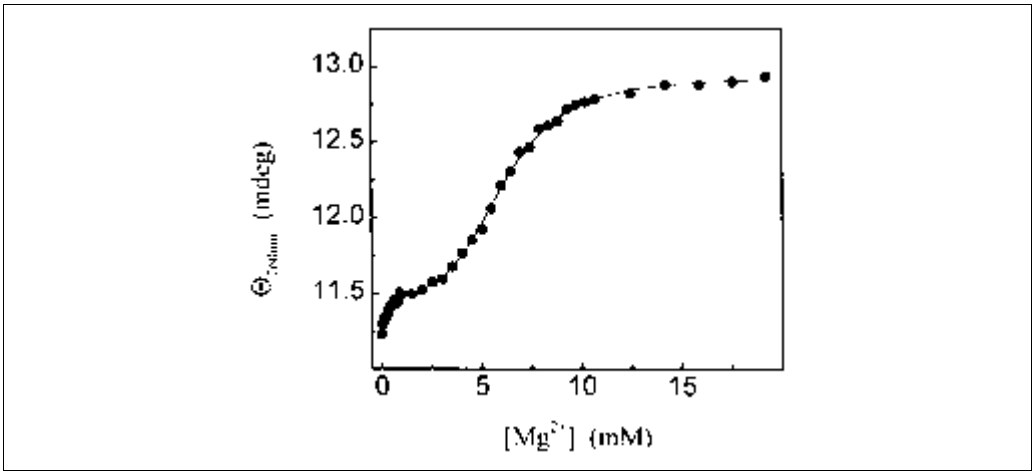

Figure 11.5.1 Sample Mg^{2+}-induced folding transition fit using Equation 11.5.4 and n_1 = 1.0, n_2 = 4, K_{D1} = 0.4 mM, and K_{D2} = 6.1 mM Mg^{2+}.

3. Record CD and absorbance (see Basic Protocol 1) at wavelengths determined with the coarse Mg^{2+} titration. Add the least volume possible of concentrated Mg^{2+} solution so that correction for dilution of the RNA will be minimal. Use multiple Mg^{2+} stock solutions (e.g., 10 mM and 1 M Mg^{2+}) to optimize coverage of each transition.

   The sensitive titration may require that over fifty separate measurements be conducted, each following the addition of small quantities (<10 μL) of the concentrated Mg^{2+} stock solutions. Rapid additions without opening the sample compartment can be made through a small-bore HPLC-style capillary tubing connected to calibrated glass syringe.

   To further increase accuracy, the glass syringe can be attached to manual push-button repeating dispenser (available from Hamilton) that can reliable dispense 1/50 of the syringe volume per increment. To further expedite the titration process, an electronically controlled titrator (also from Hamilton) can be programmed and interfaced to the spectrophotometer.

4. Correct the CD signal for dilution of RNA due to added $MgCl_2$. Fit the Mg^{2+}-induced transition between states A and B to a binding curve according to the following equation:

$$\theta(Mg^{2+}) = \theta_A + \frac{[Mg^{2+}]^n}{[Mg^{2+}]^n + (K_D)^n}(\theta_B - \theta_A)$$

Equation 11.5.2

where θ_A and θ_B are the signals of states A and B, respectively. For two well-separated transitions A→B and B→C, fit the signal as the sum of two single transitions:

$$\theta(Mg^{2+}) = \theta_A + \frac{[Mg^{2+}]^{n_1}}{[Mg^{2+}]^{n_1} + (K_{D1})^{n_1}}(\theta_B - \theta_A) + \frac{[Mg^{2+}]^{n_2}}{[Mg^{2+}]^{n_2} + (K_{D2})^{n_2}}(\theta_C - \theta_B)$$

Equation 11.5.3

where n_1 and K_{D1}, and n_2, and K_{D2} are the Hill coefficients and midpoints of the first and second transitions, respectively, and θ_A, θ_B, and θ_C are the CD signals of states A, B, and C (Fig. 11.5.1). For two overlapping transitions where all three species are significantly populated, fit the signal to reflect this linked equilibrium according to:

$$\theta(Mg^{2+}) = \frac{\theta_A + \theta_B([Mg^{2+}]/K_{D1})^{n_1} + \theta_C([Mg^{2+}]/K_{D1})^{n_1}([Mg^{2+}]/K_{D2})^{n_2}}{1 + ([Mg^{2+}]/K_{D1})^{n_1} + ([Mg^{2+}]/K_{D1})^{n_1}([Mg^{2+}]/K_{D2})^{n_2}}$$

Equation 11.5.4

Equation 11.5.4 reduces to Equation 11.5.3 when $K_{D1} << K_{D2}$.

BASIC PROTOCOL 3

MEASUREMENT AND ANALYSIS OF A UREA TITRATION

The nonionic denaturant urea can be used to denature both secondary and tertiary RNA structures and a urea titration can determine the free energy and size of a folding transition (Shelton et al., 1999). This protocol involves measuring the CD signal at the appropriate wavelength for each sample at varying urea concentrations over an extended range, e.g., 0 to 7 M. The required concentration range will depend upon the size of the RNA and must be wide enough that accurate pre- and post-transition baselines can be observed. Other solvent conditions such as Mg^{2+} concentration and pH must remain constant.

Materials

Unfolded RNA sample
Urea (ultrapure; filter through 0.2-μm filter)

Additional reagents and equipment for determining CD spectrum (see Basic Protocol 1)

Prepare urea/RNA stock solutions

1. Prepare two urea solutions, one at low (e.g., 0.7 M) and one at high (e.g., 7 M) concentration.
2. Make a stock of 10× concentrated unfolded RNA in either the low- or high-molarity urea solution.
3. Add one part of the 10× concentrated sample to nine parts of *each* of the low- and high-molarity urea solutions.

   Final RNA concentration should be such that A_{260} is equal to 0.3 to 0.8 absorbance units.

   This ensures that each urea solution contains the same RNA concentration, thereby ensuring that mixtures of these two solutions are at the same RNA concentration and that the CD signal will not need to be corrected for varying RNA concentration.

Measure CD spectra on a series of urea concentrations

4a. *Method a:* Prepare individual samples at varying urea concentrations by mixing the two stock solutions at varying ratios (see Table 11.5.1). Measure each of these samples separately (see Basic Protocol 1).

To make up 21 separate 1.5-mL samples, at least 15 mL of each stock solution are required.

Table 11.5.1 Preparation of Individual Samples for Urea Titration

Sample number	Vol. 0.7 M urea solution (mL)	Vol. 7.0 M urea solution (mL)	Final urea conc. (M)
1	1.5	0	0.7
2	1.425	0.075	1.015
3	1.35	0.15	1.33
4	1.275	0.225	1.645
5	1.2	0.3	1.96
6	1.125	0.375	2.275
7	1.05	0.45	2.59
8	0.975	0.525	2.905
9	0.9	0.6	3.22
10	0.825	0.675	3.535
11	0.75	0.75	3.85
12	0.675	0.825	4.165
13	0.6	0.9	4.48
14	0.525	0.975	4.795
15	0.45	1.05	5.11
16	0.375	1.125	5.425
17	0.3	1.2	5.74
18	0.225	1.275	6.055
19	0.15	1.35	6.37
20	0.075	1.425	6.685
21	0	1.5	7

4b. *Method b:* Prepare single sample initially containing the dilute urea solution and measure spectrum. Add small aliquots (e.g., 50 µL) of the concentrated urea solution (see Table 11.5.2) and measure spectrum after each addition (see Basic Protocol 1).

This method produces more accurate data and uses ~5- to 10-fold less sample than method a. The size of each aliquot determines the urea concentration spacing of the data. Additions through a small-bore HPLC-style capillary tubing connected to calibrated glass syringe can improve both speed and accuracy of this method.

4c. *Method c:* Perform as in step 4b, but remove a small aliquot prior to the addition of an equivalent volume aliquot (e.g., remove 50 µL, then add 50 µL) so that the total sample volume remains constant.

This variation reduces the total amount of sample required while increasing the range of the urea titration for a given number of additions (see Table 11.5.3). A two-syringe programmable titrator capable of both removal and additions can greatly facilitate the implementation of this technique.

Analyze data

5. To determine the free energy between states A and B in the absence of urea, ΔG^{o}_{H2O}, and its dependence on denaturant according to ΔG^{o} (urea) = ΔG^{o}_{H2O}, fit the data to:

$$\theta(\text{urea}) = \frac{\theta_A + \theta_B e^{(-\Delta G_{H_2O} - m^{o}[\text{urea}])/RT}}{1 + e^{(-\Delta G_{H_2O} - m^{o}[\text{urea}])/RT}}$$

Equation 11.5.5

where R is the gas constant (1.987 cal/K/mol), T is the absolute temperature (K), and θ_A and θ_B are the baseline values of states A and B, respectively (Fig. 11.5.2).

Table 11.5.2 Add-Aliquot Variation for Preparation of Samples for Urea Titration[a]

Addition number	Net vol. 7.0 M urea solution added (mL)	Final urea conc.[b] (M)
0	0	0.7
1	0.05	0.90
2	0.1	1.09
3	0.15	1.27
4	0.2	1.44
5	0.25	1.6
6	0.3	1.75
7	0.35	1.89
8	0.4	2.03
9	0.45	2.16
10	0.5	2.28
11	0.55	2.39
12	0.6	2.5
13	0.65	2.60
14	0.7	2.70
15	0.75	2.8
16	0.8	2.89
17	0.85	2.98
18	0.9	3.06
19	0.95	3.14
20	1	3.22

[a]Starting condition is 1.5 mL of 0.7 M urea.
[b]Calculated according to $C = [\text{Vol}_{\text{init}} \times (C_{\text{init}} + \text{Vol}_{\text{add}}) \times C_{\text{add}})/(\text{Vol}_{\text{init}} + \text{Vol}_{\text{add}})$, where C is the urea concentration, Vol_{init} and Vol_{add} are the initial and added volumes, and C_{init} and C_{add} are the urea concentrations of the initial and added solutions, respectively.

Table 11.5.3 Remove/Add-Aliquot Variation for Preparation of Samples for Urea Titration[a]

Addition no.	Final urea conc.[b] (M)
0	0.70
1	0.93
2	1.14
3	1.35
4	1.55
5	1.75
6	1.94
7	2.12
8	2.29
9	2.46
10	2.62
11	2.78
12	2.93
13	3.07
14	3.21
15	3.35
16	3.48
17	3.61
18	3.73
19	3.84
20	3.96

[a]Starting condition is 1.5 ml of 0.7 M urea, solution added is 7 M urea, with removal and addition in 50-µL aliquots.
[b]Calculated according to the recursive relation $C_i = [(Vol_{init} - Vol_{add}) \times C_{i-1} + Vol_{add} \times C_{add}]/Vol_{init}$, where C is the urea concentration, Vol_{init} and Vol_{add} are the initial and added volumes, and C_{i-1} and C_{add} are the urea concentrations of the previous and added solutions, respectively.

a. The baseline values may be a constant value, or may be approximated as a line, e.g., $\theta_A(\text{urea}) = m_A[\text{urea}] + \theta^{H_2O}_A$. For two transitions from A→B and B→C with stabilities $\Delta G^o_{A\rightarrow B}(\text{urea}) = \Delta G^o_{A\rightarrow B}(0) + m^o_{A\rightarrow B}[\text{urea}]$ and $\Delta G^o_{B\rightarrow C}(\text{urea}) = \Delta G^o_{B\rightarrow C}(0) + m^o_{B\rightarrow C}[\text{urea}]$, the signal can be fit according to:

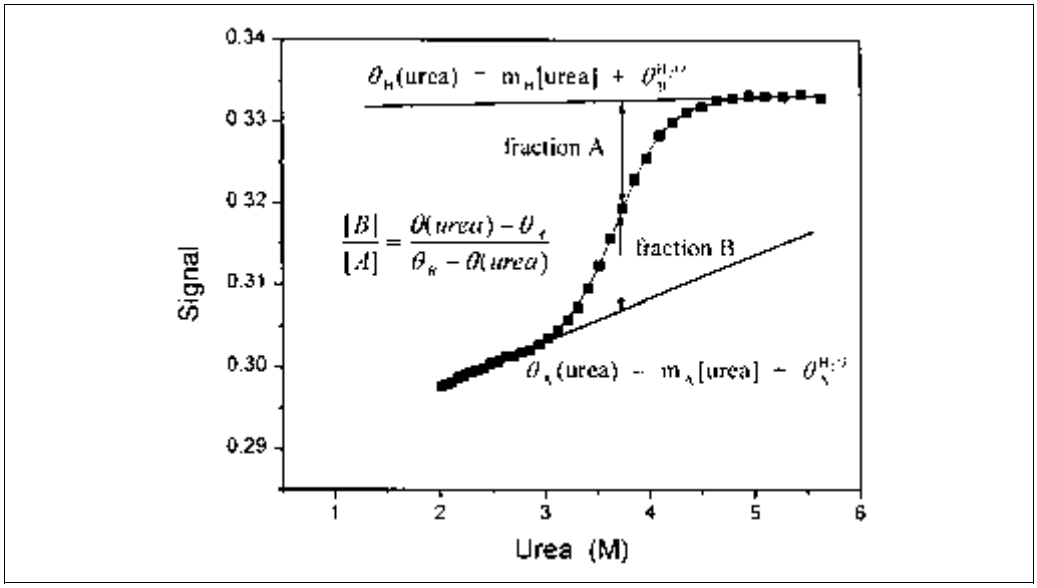

Figure 11.5.2 Sample urea denaturation profile fit using Equation 11.5.5 with ΔG^o= 9.3 kcal/mol and m^o = 2.5 kcal/mol/M.

$$\theta(\text{urea}) = \frac{\theta_A + e^{-\Delta G^o_{A\to B}(\text{urea})/RT}(\theta_B + \theta_C e^{-\Delta G^o_{B\to C}(\text{urea})/RT})}{1 + e^{-\Delta G^o_{A\to B}(\text{urea})/RT}(1 + e^{-\Delta G^o_{B\to C}(\text{urea})/RT})}$$

Equation 11.5.6

where θ_A, θ_B, and θ_C are the ellipticities of states A, B, and C (e.g., $\theta_A(\text{urea}) = m_A[\text{urea}] + \theta_A^{H_2O}$).

b. For broad transitions, it can be difficult to simultaneously fit the *m* value and a sloping baseline with confidence. An alternative method to determine the *m* value is to use multiple Mg^{2+} titrations to measure the K_D at different, fixed concentrations of urea. The change in free energy due to the addition of urea at any given Mg^{2+} concentration can be written as the difference of the stability before and after addition of urea:

$$\Delta\Delta G^o(\text{urea}) = \Delta G^o_{H_2O} - \Delta G^o(\text{urea}) = -RT \ln \left(\frac{K_D^{H_2O}}{K_D(\text{urea})} \right)^n = -nRT \ln \frac{K_D^{H_2O}}{K_D(\text{urea})}$$

Equation 11.5.7

A plot of $\Delta\Delta G^o$(urea) versus urea concentration can be fit to the linear relation shown in Equation 11.5.11.

COMMENTARY

Background Information

The characterization of the thermodynamic parameters of a tertiary RNA is fundamental to understanding its folding behavior. A common method for measuring the stability of RNAs is thermal denaturation. From thermal melting curves or differential scanning calorimetry, the enthalpy, free energy, and entropy can be determined, making these powerful tools in the study of RNA thermodynamics. However, thermal denaturation of Mg^{2+}-dependent RNAs may not be reversible due to Mg^{2+}-induced degradation at high temperatures. Further, the thermal melting of an RNA tertiary structure can be difficult to separate from the melting of the secondary structure. Consequently, other methods to measure RNA secondary and tertiary stability are desirable.

This unit presents spectroscopic methods for obtaining the Mg^{2+} midpoint and the effective number of Mg^{2+} ions bound in the transition. These properties can be used to obtain the tertiary stability of the RNA. The use of urea titrations provides an alternative to obtain these quantities, as well as a measure of the amount of surface buried in the transition, the urea *m* value.

Much of the analysis required for the proper extraction of the apparent thermodynamic parameters requires the use of an analysis program capable of nonlinear least-squares fitting. Many excellent plotting and analysis packages are commercially available (e.g., Origin by Microcal).

The basis of Equation 11.5.2, Equation 11.5.3, and Equation 11.5.4 is the following, which assumes that Mg^{2+} ions are required and that they specifically bind in the folding of a tertiary RNA. For a single transition between states A and B where *n* Mg^{2+} ions are bound cooperatively:

$$A + nMg^{2+} \overset{K_d}{\leftarrow} B$$

Equation 11.5.8

The dissociation constant, K_d, and the Mg^{2+} midpoint, K_D, can be written as:

$$K_d = (K_D)^n = \frac{[Mg^{2+}]^n [A]}{[B]}$$

Equation 11.5.9

The Mg^{2+}-dependent equilibrium free energy, ΔG^o, between states A and B is the logarithm of the ratio of their populations and can be written:

$$\Delta G^o = -RT \ln K_{eq} = -RT \ln \frac{[B]}{[A]}$$

$$= -RT \ln \left(\frac{[Mg^{2+}]}{K_D} \right)^n = -nRT \ln \frac{[Mg^{2+}]}{K_D}$$

Equation 11.5.10

where K_{eq} is the equilibrium constant between states A and B.

It should be realized that the Hill coefficient identifies the minimal number of Mg^{2+} ions involved in the transition. However, a Hill coefficient of unity can be attributed to multiple, independent site binding of approximately the same K_d, so that the total number of Mg^{2+} ions bound is unknown. This number can be determined by other methods including equilibrium dialysis.

Urea denatures RNAs by preferentially stabilizing the unfolded state; however, the precise mechanism is unknown. Urea may promote unfolding either by forming hydrogen bonds with newly exposed carbonyls and nitrogens on the bases, increasing the structure of water and making the exposure of hydrophobic regions (e.g., the aromatic rings) less favorable, or by weakly binding to the RNA, with the number of urea binding sites increasing upon unfolding (Makhatadze and Privalov, 1992).

Regardless of which modes are operational, empirically the stability of secondary and tertiary RNA structures can be well approximated with a linear dependence upon urea concentration at a fixed Mg^{2+} concentration:

$$\Delta G^{o}(\text{urea}) = \Delta G^{o}_{H_2O} - m^{o}[\text{urea}]$$

$$= -RT \ln K_{eq}(\text{urea}) = -RT \ln \frac{[B]}{[A]}$$

$$= -RT \ln \left[\frac{\theta(\text{urea}) - \theta_A}{\theta_B - \theta(\text{urea})} \right]$$

Equation 11.5.11

For proteins, the slope m^{o} is proportional to the amount of urea-sensitive surface buried upon folding and is directly related to the size of the protein (Myers et al., 1995). Ongoing studies in the author's laboratory suggest that this is true for RNAs as well (Shelton et al., 1999).

Critical Parameters and Troubleshooting

RNA is very sensitive to degradation by ribonucleases. Caution should be used to avoid contamination, including the use of latex gloves and autoclaved reagents. Sample cuvettes and other equipment such as HPLC tubing should be designated for RNA usage only, to whatever extent feasible.

Measure buffer blanks in the same cuvette orientation for proper background subtractions. With viscous urea solutions, extreme care must be taken to ensure adequate mixing of the different-density solutions. Ensure thorough mixing with a magnetic stirrer. A simple method to test for proper mixing is to confirm that the trace for dilutions going from low to high urea concentration matches that for dilutions from high to low concentration. This procedure also confirms that the folding is reversible.

Thermodynamic measurements require that the system be reversible and in complete equilibrium during the measurement. Tertiary RNA folding transitions can take from tens of minutes to hours, and may be very sensitive to experimental conditions (e.g., temperature, Mg^{2+}, and urea concentration). Slow transitions often have a high activation enthalpy and can be greatly accelerated with temperature.

Anticipated Results

These experiments require tens of micrograms of RNA per titration. From the measurement of a Mg^{2+} titration, the number of equilibrium folding transitions and their Mg^{2+} midpoints and Hill coefficients can be determined. From this information, the stability of an RNA can be determined at any Mg^{2+} concentration. Similarly, from a urea melting measurement starting from a folded RNA, the stability and the amount of urea-sensitive surface area buried (m^{o} value) in the transition can be determined at a given temperature and Mg^{2+} concentration.

Time Considerations

These measurements can be performed in a day or less. The use of an automated titrator will greatly accelerate the rate of data collection and multiple titrations can be conducted in a day.

Literature Cited

Banerjee, A.R. and Turner, D.H. 1995. The time dependence of chemical modification reveals slow steps in the folding of a group I ribozyme. *Biochemistry* 34:6504-6512.

Fang, X., Pan, T., and Sosnick, T.R. 1999. A thermodynamic framework and cooperativity in the tertiary folding of a Mg^{2+}-dependent ribozyme. *Biochemistry* 38:16840-16846.

Makhatadze, G.I. and Privalov, P.L. 1992. Protein interactions with urea and guanidinium chloride. A calorimetric study. *J. Mol. Biol* 226:491-505.

Myers, J.K., Pace, C.N., and Scholtz, J.M. 1995. Denaturant *m* values and heat capacity changes: Relation to changes in accessible surface areas of protein unfolding [published erratum appears in *Protein. Sci.* 1996 May;5(5):981]. *Protein Sci.* 4:2138-2148.

Pan, T. and Sosnick, T.R. 1997. Intermediates and kinetic traps in the folding of a large ribozyme revealed by circular dichroism and UV absorbance spectroscopies and catalytic activity. *Nat. Struct. Biol.* 4:931-938.

Sclavi, B., Woodson, S., Sullivan, M., Chance, M.R., and Brenowitz, M. 1997. Time-resolved synchrotron X-ray "footprinting," a new approach to the study of nucleic acid structure and function: Application to protein-DNA interactions and RNA folding. *J. Mol. Biol.* 266:144-159.

Shelton, V., Sosnick, T.R, and Pan, T. 1999. Applicability of urea in the thermodynamic analysis of secondary and tertiary RNA folding. *Biochemistry* 38:16831-16839.

Zarrinkar, P.P. and Williamson, J.R. 1994. Kinetic intermediates in RNA folding. *Science* 265:918-924.

Contributed by Tobin R. Sosnick
University of Chicago
Chicago, Illinois