UV Thermal Melting Curves of $tRNA^{Phe}$ in the Presence of Ligands

Sarah R. Kirk,* Todd P. Silverstein, and Karen L. McFarlane Holman
Department of Chemistry, Willamette University, Salem, OR 97301; *skirk@willamette.edu

UV–vis spectrophotometry can be used to assay the three-dimensional conformation of biological molecules *(1, 2)*. Because of the positive entropy of unfolding, native secondary and tertiary structures of $tRNA^{Phe}$ are only stable below about 50–70 °C *(3)*. We have designed a laboratory project to investigate, via UV thermal melting curves, the influence of equilibrium ligand binding on the conformational stability of $tRNA^{Phe}$. This project is a part of an integrated biochemistry laboratory course that probes conformational changes in $tRNA^{Phe}$ using a variety of biochemical and biophysical methods *(4)*.

The temperature at which the folded and unfolded forms of $tRNA^{Phe}$ are in a 50/50 equilibrium, T_{melt}, is determined by monitoring UV absorbance over a range of temperatures *(5)*. In the following set of laboratory experiments, T_{melt} is determined for $tRNA^{Phe}$ bound to various small molecule ligands. Ligands are selected to assess the importance of different binding modes between ligands and $tRNA^{Phe}$ (e.g., aromatic ring π-stacking, ionic bonding, hydrogen bonding, or electrostatic screening). Mg^{2+} and small molecule cationic ligands such as spermine and spermidine have been shown to be necessary for proper folding of $tRNA^{Phe}$ *(6)*. It has been reported that aminoglycoside antibiotics such as kanamycin A and neomycin B bind electrostatically to RNA, displacing magnesium ions *(7)*, stabilizing the folded form and causing an increase in T_{melt}. While ethidium bromide binds through intercalation *(8)*, it also increases the stability of folded $tRNA^{Phe}$ and raises T_{melt}. These experiments provide an opportunity to monitor the effects of concentration, net positive charge, and binding modes on the structural stability of $tRNA^{Phe}$.

Stock Solutions and Equipment

$tRNA^{Phe}$ from brewers yeast is purchased from Sigma-Aldrich (R 4018), diluted to 1 mg/mL (40 μM), and stored at −20 °C. The 5× reaction buffer stock is prepared with the following concentrations: 250 mM Tris·HCl (pH 7.5) and 500 mM NaCl. Ethidium bromide is prepared as a 5.0 mg/mL stock solution. Small molecule ligands are available from Sigma-Aldrich and are prepared as the following stock solutions: neomycin B (0.5 M), spermine (1 M), spermidine (1 M), and kanamycin A (0.25 M).

A Cary 3 UV–vis spectrophotometer equipped with a temperature controller is used to measure the thermal melting curves of $tRNA^{Phe}$. A multicell block allows us to measure six samples concurrently. tRNA absorbance is monitored at 260 nm, and melting curves are measured using the Cary 3 program "Thermal". Temperature is varied from 20 °C to 90 °C at a rate of 0.5 °C/min. Reversibility is assayed by running 4 ramps overnight: ramp 1 (initial heating 20 °C → 90 °C), ramp 2 (cooling from 90 °C back down to 20 °C), ramp 3 (second heating from 20 °C → 90 °C), and ramp 4 (second cooling back to 20 °C).

Typical Procedure

Six 1.6 mL samples containing 1 μM $tRNA^{Phe}$ are prepared in a 100 mM NaCl/50 mM Tris, pH 7.5 buffer. After the addition of small molecule ligands, samples are heated to 90 °C for 30 seconds to unfold the $tRNA^{Phe}$ and subsequently cooled to room temperature over 15 minutes to allow refolding in the presence of the ligands. The 1.6 mL samples are placed in quartz semi-micro cuvettes capped with Teflon stoppers. Typical runs include 1 μM $tRNA^{Phe}$ (a) alone (control); (b) with Mg^{2+}; (c) with two different concentrations of a small molecule ligand; and (d) with two different concentrations of EtBr. In the second part of this project, the T_{melt} is determined for the $tRNA^{Phe}$–ligand complex at five more concentrations of the small molecule ligand; concentration ranges are given in Table 1.

Data Analysis

The melting temperature is determined by calculating the derivatives of the absorbance versus temperature curve using the Cary program "Thermal". Averaged over the four ramps, T_{melt} is plotted versus the ligand concentration using KaleidaGraph software. These data are fit to a hyperbolic saturation curve using K_d, the equilibrium dissociation constant for the tRNA–ligand complex, as a fittable parameter (see eq 2 in ref *4*). Using their own data and pooled class data, students determine the K_d for binding of Mg^{2+}, EtBr, and the small molecule ligands to $tRNA^{Phe}$. They can then draw conclusions as to the relative importance of net charge, intercalation, and hydrogen bonding in the tRNA–ligand binding interaction. Typical results are included in our article describing the full laboratory course *(4)*.

Hazards

Ethidium bromide is a mutagen. To minimize student exposure to the hazardous powder, a stock solution of ethidium bromide is prepared by our laboratory technician. The solution is

Table 1. Suggested Concentration Ranges for Ligands

Ligand	[Ligand] Range
Mg^{2+}	0.1–10 mM
Spermine	250–2500 μM
Spermidine	500–5000 μM
Neomycin B	1–100 μM
Kanamycin A	20–8000 μM
EtBr	1–50 μM

prepared at the lowest possible concentration, to minimize both exposure and waste. Students wear safety gloves and glasses at all times. In the unlikely event that solutions come in contact with skin or body, the affected area should be flushed with water immediately. EtBr solutions are evaporated and disposed as hazardous waste. For more details on safety precautions, see ref *4*.

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