Fluorescence Spectroscopy of tRNA^{Phe} Y Base in the Presence of Mg^{2+} and Small Molecule Ligands

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We have designed a laboratory unit focused on ligand-induced changes in tRNA^{Phe} fluorescence. This unit is part of a larger integrated biochemistry laboratory course probing conformational changes in tRNA^{Phe} using a variety of biochemical and biophysical methods *(1)*. tRNA^{Phe} contains a unique Y base that consists of three planar aromatic rings and serves as a fluorophore (Figure 1). Because Y base fluorescence is exquisitely sensitive to the local environment, it serves as an intrinsic probe to study the conformational changes of tRNA^{Phe} in the presence of magnesium ions and small molecule ligands *(2–4)*.

Most polycations (e.g., neomycin B, spermine) bind to the major groove in tRNA^{Phe} and stabilize a stacked-in conformation of the Y base *(4)*. In contrast, the aromatic monocation ethidium bromide (EtBr; Figure 1) binds to tRNA by intercalating into its central π-stack. When EtBr binds to tRNA^{Phe}, it tends to stabilize a "flipped-out" conformation for the Y base *(5)*. Furthermore, EtBr is also fluorescent and can be used to independently monitor ligand binding to tRNA as well as to DNA *(6)*. The EtBr fluorescence intensity is higher in the EtBr–tRNA complex relative to aqueous EtBr *(7)*.

In the first experiment described here, students use fluorescence spectrophotometry to probe structural changes within the tRNA^{Phe} molecule by monitoring Y base fluorescence as Mg^{2+} or a small molecule ligand (e.g., neomycin B, spermine) is titrated into a solution of tRNA^{Phe}. In the second experiment, changes in Y base fluorescence are followed as EtBr is titrated into a tRNA^{Phe} solution. Subsequently, the EtBr and Y base fluorescence intensities are monitored as a small molecule is added.

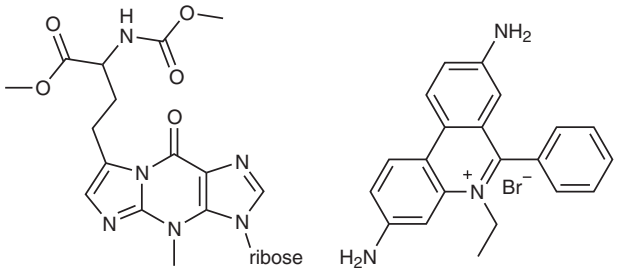

Figure 1. The Y base nucleotide (left) and ethidium bromide (right).

Table 1. Fluorimeter Parameters for Y Base and Ethidium Bromide

Fluorescence Marker	λ_{ex}/nm	λ_{em}/nm	Slit Widths (ex/em)	Scan Speed
tRNA^{Phe} Y base	318	380–460	20 nm/20 nm	slow
Ethidium bromide	546	580–620	10 nm/10 nm	slow

NOTE: em is emission and ex is excitation.

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), 500 mM NaCl, and 50 mM $MgCl_2$. Ethidium bromide is prepared as a 5.0 mg/mL stock solution. Small molecule ligands (neomycin B, spermine, spermidine, and kanamycin A) are available from Sigma-Aldrich.

Fluorimetry is conducted with a Cary Eclipse fluorescence spectrophotometer using the parameters listed in Table 1. The concentration of tRNA^{Phe} is confirmed by measuring the absorbance at 260 nm (ε_{260} = 371,000 M^{-1} cm^{-1}).

Typical Procedure

Three 1.00 mL samples of tRNA^{Phe} (2 μM) in 100 mM NaCl/50 mM Tris·HCl buffer, pH 7.5, are prepared. In week 1 of the experiment, Mg^{2+} is titrated into a sample of tRNA^{Phe} and Y base fluorescence is monitored. Then, to a second sample of tRNA^{Phe}, a small molecule ligand (either spermine, spermidine, kanamycin A, or neomycin B) is titrated while monitoring Y base fluorescence. The third tRNA^{Phe} sample is used to zero the fluorimeter, and a fourth buffer-only control sample is titrated to ascertain background titrant fluorescence.

Stock solutions of the titrating reagent (small molecule ligand or Mg^{2+}) are prepared so that titrant aliquots are small (2–4 μL) and the total volume of 1.0 mL is not significantly altered. Before each fluorescence scan, titrant aliquots are added and the stoppered cuvette is inverted at least three times. Each student group separately titrates Mg^{2+} and one of the small molecule ligands over the concentration ranges given in Table 2.

During week 2 of the experiment, both the Y base and EtBr serve as fluorophores. Again two experiments are carried out, but this time they are performed in a single cuvette containing 1.00 mL tRNA^{Phe} (2 μM) in 100 mM NaCl/50 mM Tris·HCl buffer, pH 7.5.[1] In the first step of the experiment, EtBr is titrated into tRNA^{Phe} and Y base fluorescence is monitored. When Y base fluorescence intensity reaches a maximum during the titration, EtBr binding to tRNA^{Phe} is saturated. At this point, a small molecule ligand is titrated into the resulting tRNA^{Phe}–EtBr mixture. Both Y base and EtBr fluorescence are monitored after addition of each aliquot of ligand. Each group titrates tRNA^{Phe} with EtBr, followed by one of the small molecule ligands. Students use the same ligand as in the previous week's experiment; concentration ranges are given in Table 3.

Data Analysis

A graph of Y base fluorescence intensity versus ligand concentration is generated for each of the titrations. Fluorescence should saturate with ligand concentration; thus, students

Table 2. Suggested Concentration Ranges for Ligands

Ligand	[Ligand] Range	Notes
Mg^{2+}	0–4 mM	In the 0–0.04 mM range, collect data every 0.01 mM. In the 0.04–0.12 mM range, collect data every 0.02 mM. In the 0.12–4 mM range, collect five or six more data points.
Spermine	0–1200 μM	In the 0–200 μM range, collect data every 50 μM. In the 200–1200 μM range, collect data every 200 μM.
Spermidine	0–2000 μM	In the 0–200 μM range, collect data every 50 μM. In the 200–2000 μM range, collect data at 500 μM and every 500 μM thereafter.
Kanamycin A	0–850 μM	In the 0–250 μM range, collect data every 50 μM. In the 250–850 μM range, collect data every 100 μM.
Neomycin B	0–300 μM	In the 0–100 μM range, collect data every 20 μM. In the 100–300 μM range, collect data every 50 μM.

Table 3. Suggested Concentration Ranges for Ligands

Ligand	[Ligand] Range	Notes
Ethidium bromide	0–50 μM	In the 0–10 μM range, collect data every 2 μM. In the 10–50 μM range, collect data every 10 μM.
Spermine	0–2000 μM	In the 0–200 μM range, collect data every 50 μM. In the 200–2000 μM range, collect data every 200 μM.
Spermidine	0–2000 μM	In the 0–200 μM range, collect data every 50 μM. In the 200–2000 μM range, collect data every 200 μM.
Kanamycin A	0–850 μM	In the 0–250 μM range, collect data every 50 μM. In the 250–850 μM range, collect data every 100 μM.
Neomycin B	0–10 μM	In the 0–2 μM range, collect data every 0.1 μM. In the 2–10 μM range, collect data every 2 μM.

determine K_d, the equilibrium dissociation constant for the tRNA–ligand complex by fitting the fluorescence titration data to a hyperbolic saturation curve (see eq 2 in ref *1*).

Further titration of the saturated solution of the $tRNA^{Phe}$–EtBr complex with other small ligands displaces EtBr from its $tRNA^{Phe}$ binding sites, thereby causing changes in both Y base and EtBr fluorescence. Students assess this phenomenon by monitoring both the Y base fluorescence of the $tRNA^{Phe}$ and the fluorescence of EtBr as it is released into solution. Fitting the data to hyperbolic satuation curves,[2] students compare K_d values for the different small molecule ligands, Mg^{2+}, and EtBr. They then discuss the relative importance of different types of binding interactions: charge–charge attraction, charge–dipole attraction, hydrogen bonding, and intercalation. Typical results are included in our article describing the full laboratory course *(1)*.

Hazards

Ethidium bromide is a mutagen. To minimize student exposure to solid ethidium bromide, a stock solution is prepared by our laboratory technician. The solution is 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. Solutions containing EtBr are collected, evaporated, and treated as outlined in ref *1*.

Notes

1. As before, a second $tRNA^{Phe}$ sample is used to zero the fluorimeter, and a third buffer-only control sample is titrated to ascertain titrant background fluorescence.

2. Data that decrease hyperbolically are fit to eq 1 in ref *1,* whereas data that rise hyperbolically are fit to eq 2 in ref *1*.

Literature Cited

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