Metal-Catalyzed Cleavage of tRNA^{Phe}

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Lead(II)-catalyzed hydrolysis is an effective assay of tRNA^{Phe} tertiary structure *(1, 2)*. Lead(II) catalyzes the cleavage of native, folded tRNA^{Phe} at a single site in the D loop resulting in a large fragment and a small fragment *(3)*, as depicted in Figure 1. Ligand binding alters the conformation of tRNA^{Phe}, thereby inhibiting lead-catalyzed cleavage *(4)*; metals other than lead may also cleave tRNA *(5)*. This laboratory project uses polyacrylamide gel electrophoresis (PAGE) to assay tRNA^{Phe} hydrolysis in three experiments that explore cleavage of tRNA^{Phe} in the presence of Pb^{2+}, Pb^{2+} plus a small molecule ligand, and metal ions other than Pb^{2+}. This project is a part of an integrated biochemistry laboratory course probing conformational changes in tRNA^{Phe} using a variety of biochemical and biophysical methods *(4)*.

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 $MgCl_2$. Stock solutions of 5.0 mg/mL ethidium bromide and 10% acrylamide in 8 M urea/1× TBE[1] are prepared once and used throughout the entire semester. Acrylamide is purchased as a 40% solution of 19:1 acrylamide:bisacrylamide. A 400 mM stock solution of lead(II) acetate is prepared fresh daily. 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). Electrophoresis is carried out using a Whatman Biometra Model V16-2 Gel Apparatus connected to a Thermo EC 400P power supply.

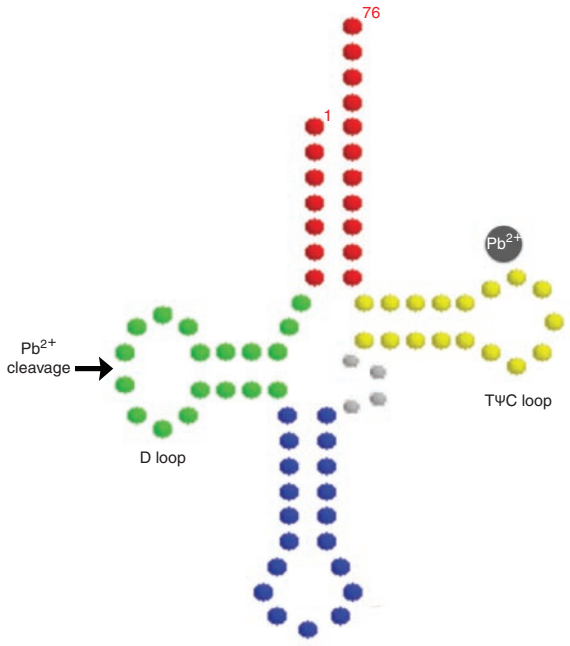

Figure 1. Secondary structure of tRNA^{Phe} showing the Pb^{2+}-binding site in the TΨC loop. Upon folding into the tertiary structure, the TΨC and D loops approach each other facilitating Pb^{2+}-catalyzed cleavage in the D loop.

Typical Procedure

All reactions are run in Eppendorf tubes (0.6 or 1.5 mL tubes) containing 16 µM tRNA^{Phe} in 1× reaction buffer. Small molecule ligands are added where appropriate. Reactions are initiated upon addition of a metal ion. The final reaction volume is brought to 10 µL with deionized water.

Experiment 1: Lead(II)-Catalyzed Hydrolysis of tRNA^{Phe}

In this experiment, students vary both Pb^{2+} concentration and incubation time. They prepare appropriate dilutions of stock 400 mM lead(II) acetate to reach final concentrations of 100–1600 µM Pb^{2+}. Tube 1 is the tRNA-only control and tubes 2–6 contain five concentrations of Pb^{2+} (100–1600 µM). Tubes 7–11 all contain the same [Pb^{2+}], either 200, 400, 600, or 800 µM Pb^{2+}, depending on the research team.

Appropriate volumes of tRNA^{Phe}, 5× reaction buffer, and deionized H_2O are added to all eleven tubes, which are then vortexed and centrifuged. Pb^{2+} is added to initiate cleavage. Incubation times are as follows: tubes 2–6, 60 minutes; tube 7 is quenched immediately; tube 8, 15 minutes; tube 9, 30 minutes; tube 10, 45 minutes; and tube 11, 60 minutes. Reactions are quenched by adding 30 µL of the loading dye (0.1 mg/mL bromophenol blue dissolved in 8 M urea and 1× TBE) and freezing at −20 °C.

Gels are crosslinked by adding 300 µL of 20% ammonium persulfate and 24 µL of TEMED[2] to 60 mL of 10% polyacrylamide. Gels are pre-electrophoresed in 1× TBE for at least 30 minutes. Sample tubes are heated for 30 seconds at 90 °C, vortexed, centrifuged, and loaded on a pre-electrophoresed gel.

Gels are electrophoresed at a fixed current of 30 mA per gel with initial settings of 500 V and 300 W. The gels are run until the tracking dye is 2 cm from the bottom. The gels are subsequently disassembled and stained with 1.0 µg/mL ethidium bromide and then visualized and quantified using a Gel Doc system. Upon evaluation of class results, students select the lead(II) concentration and incubation time that gives 50% cleavage of tRNA^{Phe}. These two parameters are used in subsequent lead(II) cleavage experiments.

Experiment 2: Lead(II)-Catalyzed Cleavage of $tRNA^{Phe}$ in the Presence of a Small Molecule Ligand

Students explore the effect of increasing concentrations of four different ligands on lead(II)-catalyzed hydrolysis of $tRNA^{Phe}$ using the specified concentration ranges given in Table 1.

Twelve Eppendorf tubes (0.6 or 1.5 mL tubes) contain 16 μM $tRNA^{Phe}$, 1× reaction buffer, and varying concentrations of small molecule ligands. The tubes are vortexed and then centrifuged to return all liquid to the bottom of the tube. Samples are heated to 90 °C for 30 seconds to unfold the $tRNA^{Phe}$. The solution is cooled to room temperature over 15 minutes to allow refolding in the presence of the ligand.

Cleavage is initiated by adding the predetermined concentration of Pb^{2+}. After the time observed to produce 50% cleavage in experiment 1, reactions are quenched, electrophoresed, stained, and analyzed.

Experiment 3: Metal Ion Catalyzed Cleavage of $tRNA^{Phe}$

Students design an experiment to test the effect of increasing concentrations of various metal ions on $tRNA^{Phe}$ hydrolysis. Each group runs two controls: tRNA-only and tRNA plus Pb^{2+}. The remaining lanes are used to study two of the eight metals listed in Table 2.

Twenty Eppendorf tubes contain 16 μM $tRNA^{Phe}$, 1× reaction buffer, and various concentrations of the two metals. The tubes are vortexed and centrifuged prior to the addition of metal ions. Tubes 1 and 19 contain $tRNA^{Phe}$ only (no metal). Tubes 2 and 20 contain $tRNA^{Phe}$ plus Pb^{2+} using the concentration and cleavage times determined in experiment 1. Tubes 3–10 and 11–18 contain varying concentrations of two different metals. Six of the eight concentrations are selected from the lower half of the range suggested in Table 2 for each metal. Tubes 3–18 are initiated by the addition of the metal ions and cleaved for 16–20 hours. Reactions are quenched, run on PAGE, stained, and analyzed according to instructions in experiment 1 above.

Data Analysis

PAGE results are analyzed using Biorad Gel Doc 1000 with Quantity One software. Bands representing the full length $tRNA^{Phe}$ and the largest fragment are identified and quantified. Percent cleavage (fragment divided by total tRNA) is calculated for each lane and plotted as % cleavage versus ligand concentration. Where appropriate, results 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 1 in ref *4*). The class compares data for metal-catalyzed cleavage in the presence of various small molecule ligands and metal cations. Students discuss cleavage catalyzed by different metals in light of concepts from inorganic chemistry such as charge–radius ratio, hard–soft acid–base theory, structure of the primary coordination sphere, and so forth. By comparing K_d values for different small molecule ligands, students draw conclusions on the importance of net charge and hydrogen bonding in the tRNA–ligand binding. Typical results and conclusions are included in our manuscript describing the full laboratory course *(4)*.

Table 1. Suggested Concentration Ranges for Ligands

Small Molecule Ligand	Range
Neomycin B	0.10–50 mM
Spermine	0.10–100 mM
Spermidine	0.10–200 mM
Kanamycin A	0.10–75 mM

Table 2. Suggested Concentration Ranges for Metal Ions

Metal Ion	Salt	Range
Eu^{3+}	$EuCl_3 \cdot 6H_2O$	2–1000 μM
Mg^{2+}	$MgCl_2 \cdot 6H_2O$	5–1500 mM
Gd^{3+}	$GdCl_3 \cdot 6H_2O$	2–1000 μM
Zn^{2+}	$ZnBr_2$	10–2000 μM
Mn^{2+}	$MnSO_4 \cdot H_2O$	1–200 mM
Fe^{3+}	$Fe(NO_3)_3 \cdot 9H_2O$	0.5–15 mM
Sm^{3+}	$Sm(NO_3)_3 \cdot 6H_2O$	2–1500 μM
Sn^{2+}	$SnBr_2$	50–3000 μM

Hazards

This lab uses salts and polyamines that are mild to moderate irritants. Lead(II) acetate and acrylamide are carcinogens and ethidium bromide is a mutagen. Students wear safety goggles and glasses at all times and our laboratory technician prepares several of the stock solutions. Details of all safety precautions, hazards, and waste disposal are described in ref *4*.

Notes

1. TBE (Tris-borate-EDTA) is purchased from Fisher Bioreagents as 10× stock solution: 0.89 M Tris base, 0.89 M boric acid, 0.02 M EDTA.
2. TEMED is *N*,*N*,*N*′,*N*′-tetramethylethylenediamine.

Literature Cited

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