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Inner-sphere Coordination of Divalent Metal Ion with Nucleobase in Catalytic RNA

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Abstract

Identification of the function of metal ions and the RNA moieties, particularly nucleobases, that bind metal ions are important questions in RNA catalysis. Here we combine single-atom and abasic substitutions to probe functions of conserved nucleobases in ribonuclease P (RNase P). Structural and biophysical studies of bacterial RNase P propose direct coordination of metal ions by the nucleobases of conserved uridine and guanosine in helix P4 of the RNA subunit (P RNA). To biochemically probe the function of metal ion interactions, we substituted the universally conserved bulged uridine (U51) in the P4 helix of circularly permuted *Bacillus subtilis* P RNA with 4-thiouridine, 4-deoxyuridine and abasic modifications and G378/379 with 2-aminopurine, N7-deazaguanosine, and 6-thioguanosine. The functional group modifications of U51 decrease RNase P-catalyzed phosphodiester bond cleavage 16- to 23-fold, as measured by the single-turnover cleavage rate constant. The activity of the 4-thiouridine RNase P is partially rescued by addition of Cd(II) or Mn(II) ions. This is the first time a metal-rescue experiment provides evidence for inner-sphere divalent metal ion coordination with a nucleobase. Modifications of G379 modestly decrease the cleavage activity of RNase P, suggesting outer-sphere coordination of O6 on G379 to a metal ion. These data provide biochemical evidence for catalytically important interactions of the P4 helix of P RNA with metal ions, demonstrating that the bulged uridine coordinates at least one catalytic metal ion through an inner-sphere interaction. The combination of single-atom and abasic nucleotide substitutions provides a powerful strategy to probe functions of conserved nucleobases in large RNAs.

Graphical Abstract

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Notes

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Supporting Information

Supporting data figures and tables. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

Metal ions play important structural, catalytic, and co-catalytic roles in ribozyme-catalyzed reactions.¹ The majority of metal ions interact electrostatically with the negatively charged phosphate backbone to stabilize the complex three-dimensional structure of RNA.² Additionally, a small number of specifically bound metal ions are coordinated by functional groups in the ribozyme to stabilize substrate binding, catalytic transition states and/or conformational change steps during the catalytic cycle.^{3–7} Both structural and biochemical data are important for identifying functionally relevant metal sites and unraveling their roles in the catalytic mechanism of ribozymes.^{5,6,8–12} Metal ions are either directly coordinated by functional groups (inner-sphere coordination), or are coordinated through water-mediated interactions (outer-sphere coordination).²

Inner-sphere metal ion coordination by non-bridging phosphodiester oxygen of the RNA backbone has been well studied by structural and biochemical methods including phosphorothioate substitution and metal-ion rescue.^{4,8} In contrast, the observation of metal ion coordination by nucleobases has been limited particularly for catalytic RNAs^{13–15}; structural and biochemical studies have been carried out for inner-sphere monovalent ions^{10,16}, outer-sphere divalent ions^{17–20}, and one recent observation of an inner-sphere divalent metal ion in a small, self-cleaving ribozyme.²¹ A recent structural survey finds that the second largest groups of sites, besides the phosphate backbone, that form inner-sphere interactions with Mg(II) in RNA crystal structures are the more than 1300 observed interactions with O4 of U and O6 of G.²² However, the biochemical functions of this abundant group of RNA nucleobase-Mg(II) interactions remain poorly understood.

We sought to investigate coordination of divalent metal ions to conserved nucleobases in a large ribozyme in bacterial Ribonuclease P (RNase P) to better understand the function of nucleobases in activating metal-dependent catalysis by RNA (Figure 1). RNase P is a divalent metal ion dependent endonuclease that catalyzes hydrolysis of a specific phosphodiester bond in precursor tRNA (pre-tRNA) to remove the 5' leader sequence.^{23–25} RNase P in most organisms contains one catalytic RNA subunit (P RNA) and variable numbers of protein subunits.^{26–30} The catalytic activity of RNase P is activated by divalent metal ions such as Mg(II) ions. A large number of (>150) Mg(II) ions electrostatically associate with RNase P^{31–33} but only a handful of these Mg(II) ions form specific contacts with functional groups.^{33,34} Kinetic studies of Mg(II) activation of the RNase P•pre-tRNA (ES) complex suggest that at least two classes of inner-sphere metal ions activate *B. subtilis* RNase P (Scheme 1).³⁵ One class of high affinity metal ion stabilizes an active ES*

conformation and another class of lower affinity metal ion activates cleavage. However, the binding sites for these essential metal ions and their explicit contributions to RNase P structure and function are still being defined.

In P RNA, helix P4 (Figure 1A) is the most highly conserved region³⁶ and is critical for binding metal ions important for catalytic activity.^{37,38} A number of possible metal ion binding sites near residues A48, A49, G50, G378 and G379 (*B. subtilis* numbering) have been identified by anomalous scattering in diffraction analysis of heavy metal ions soaked into RNA crystals of catalytic domain from *B. stearothermophilus* RNase P.³⁹ Metal soaks of a crystal structure of the *T. maritima* RNase P•tRNA complex led to the proposal that two catalytic metal ions bind near A49, G50 and U51 in helix P4 as part of the active site¹⁵ (Figure S1). Phosphorothioate substitution in both *E. coli* and *B. subtilis* RNase P RNA suggested that the non-bridging oxygen atoms in the phosphate backbone of A49 and G50 (*B. subtilis* numbering) are inner-sphere metal ligands.^{40–42} Based on these biochemical and structural data, a two-metal-ion mechanism was proposed in which the carbonyl oxygen-4 (O4) of the conserved bulged uridine in helix P4 is hypothesized to directly coordinate a catalytic metal ion. Nuclear magnetic resonance (NMR) and X-ray absorption spectroscopy (XAS) methods were combined to probe RNA-metal binding to a P4 stem-loop mimic of *B. subtilis* RNase P, proposing metal ion binding near residues G378 and G379.^{43,44} Modeling of this site suggested that O6 and N7 of G378/G379 contact metal ions. However, these proposed metal binding sites on nucleobases have not been biochemically characterized. Here, we combined nucleotide substitutions and mechanistic studies to directly examine whether the nucleobase of conserved U51, G378 or G379 coordinates a catalytic metal ion in *B. subtilis* RNase P RNA (Figure 1).

EXPERIMENTAL METHODS

Materials and Reagents

All nucleotide triphosphates and other chemicals were purchased from Sigma at the highest purity (RNase and DNase free grade) unless otherwise specified. Sodium dodecyl sulfate (SDS) was purchased from Fisher Scientific. Inorganic pyrophosphatase was purchased from Roche Applied Science. Ultra-pure urea was from MP Biomedicals. 5-Iodoacetamido-fluorescein (5-IAF) was obtained from Invitrogen (now Thermo Fisher Scientific) and dissolved in anhydrous DMSO (Sigma). Guanosine 5' monothiophosphate (GMPS) was synthesized from 2', 3' iso-propylidene-guanosine and thiophosphoryl chloride, as described.⁴⁶ His₆-T7 RNA polymerase was purified using Ni-NTA chromatography, as described previously using a plasmid (pRC9) kindly provided by W. T. McAllister.⁴⁷ Buffer solutions were prepared by Milli-Q (Millipore Corporation) treated deionized water and sterilized either by autoclave (inorganic buffers such as MgCl₂, CaCl₂ and KCl) or filtration (EDTA stock solution and solutions containing MES) using Stericup filter units (Millipore Corporation). Tris/MES stock solutions at various pH values were made at room temperature and the pH was re-measured at 37°C. All DNA oligonucleotides were purchased from Integrated DNA Technologies (IDT). Ribooligonucleotides containing atomic modifications at uridine or guanosine were purchased from different vendors and deprotected according to manufacturing procedure before use. The ribooligonucleotides containing 4-thiouridine

(4SU), 2-aminopurine nucleoside (2AP), N7-deazaguanosine (7deNG) and 6-thioguanosine (6SG) were purchased from Dharmacon (now GE Lifesciences). The ribooligonucleotides with 4-deoxyuridine (4deOU) and rSpacer (abasic) modifications were synthesized by the Keck Oligo Synthesis Resource (Yale School of Medicine).

Preparation of P protein, substrate and unmodified RNA

B. subtilis RNase P protein was expressed in BL21(DE3)pLysS cells containing a plasmid encoding the P protein (pPWT1) and purified as previously described.^{48,49} The *B. subtilis* Fl-pre-tRNA^{Asp} substrate containing a 5-nucleotide leader sequence was prepared as described previously^{50,51} by 5' monothiophosphate guanosine (5' GMPS) primed *in vitro* runoff transcription (4 mM ATP, GMPS, CTP, UTP, and 0.8 mM GTP in 50 mM Tris-HCl pH 8, 1 mM spermidine, 5 mM dithiothreitol (DTT) and 20 mM MgCl₂ for 6–12 hrs at 37°C) using T7 RNA polymerase with a DNA template (ptR5) linearized by BstNI restriction enzyme⁵⁰ (New England Biolabs, NEB). The transcribed 5'-GMPS-pre-tRNA was buffer exchanged and concentrated into a degassed labeling buffer (10 mM Tris-HCl, pH 7.2 and 1 mM EDTA) for reaction with 5-IAF at 37 °C overnight. The ³²P 5' end labeled pre-tRNA substrate was prepared by incubating regularly transcribed (4 mM rNTPs and same conditions as above) pre-tRNA with calf intestinal alkaline phosphatase (NEB) followed by 5' end-labeling using T4 polynucleotide kinase (NEB) and [γ -³²P] ATP according to manufacturing instructions and purified by denaturing PAGE followed by phenol/chloroform extraction and ethanol precipitation.⁵² The wild-type and U51 P RNA were transcribed *in vitro* by T7 polymerase using DNA templates (pDeltaU51) linearized by DraI restriction enzyme (NEB). All RNAs were purified by electrophoresis on a 6% (P RNA) or 10–12% (pre-tRNA) polyacrylamide/bis denaturing gel containing 7 M urea followed by buffer-exchange and concentration using Amicon Ultra (10K MWCO) and ethanol precipitation for storage at –80 °C.

Preparation of circularly permuted P RNA

Plasmids containing DNA template for transcribing circularly permuted⁵³ P RNAs (56P and 386P, plasmids named as p56P and p386P) were cloned from the p2PRNA plasmid containing tandem repeats of RNase P RNA as described before.⁴¹ The p386P plasmid was prepared using plasmid p2PRNA as the template for PCR amplification (Primers: 5'-TAA TAC GAC TCA CTA TAG AAC GTT AGA CCA CTT ACA TTT G-3'; 5'-ATG GAG ATA TCG TAC TGC AAA CGG CTC-3') to create a DNA fragment beginning with a T7 RNA promoter followed by P RNA nucleotide G386 and ending with nucleotide U365 then followed by the sequence recognized by the restriction enzyme EcoRV. This DNA fragment was then subcloned into plasmid pUC18 at the blunt site created by digestion with SmaI (New England Biolabs) to create plasmid p386P. In both 56P and 386P RNA plasmids, the native 3' and 5' termini of the P RNA molecule are joined by the sequence 3'-ACA UUU-5'.⁴¹ Following the linearization of the DNA templates by KpnI (p46P), BsaI (p56P) or EcoRV (p386) restriction enzymes (NEB) the circulated permuted P RNAs were transcribed *in vitro* as a 5' guanosine monophosphate primed (5'-GMP) RNA as previously reported.⁴¹

Preparation of modified P RNA by ligation

For substituting U51 modifications, the 10-mer or ribooligonucleotide containing the same sequence as the 5' end strand of the P4 helix (5'-GAA AGU*51 CCA U-3') containing uridine modifications (indicated by the asterisk sign and numbering) was ligated to the 5'-GMP transcribed 56P (Figure 1) RNA in the presence of a DNA splint that is complementary to all 10-nt of the RNA oligo and the first 23 or 41 nt of 56P RNA⁴¹ (5'-GGG CAT CTC AGC ACC GTG CGA GCA TGG ACT TTC A-3'⁴¹ or 5'-GCT AGG CAC GAA CAC TAC GGG CAT CTC AGC ACC GTG CGA GCA TGG ACT TTC -3'). For G378/379 modifications, the 20-mer ribooligonucleotide containing the same sequence as the 3' end strand of the P4 helix (5'-GGA ACA AAA CAU G*378G*379C UUA CA-3') with guanosine modifications was ligated to the 5'-GMP transcribed 386P RNA in the presence of a DNA splint that is complementary to all 20-nt of the RNA oligo and the first 56 nt of 386P RNA (5'-CAA GAT CTG CAG CGA TTA CCC GAA CGT TAA GAA CAA ATG TAA GTG GTC TAA CGT TCT GTA AGC CAT GTT TTG TTC C-3').

For ligation reactions, 4 μ M 5'-GMP P RNA, 28 μ M 10-mer or 20-mer ribooligonucleotide and 28 μ M DNA splint were annealed in 66 mM Tris-HCl (pH 7.6), 6.6 mM MgCl₂ and 10 mM DTT by denaturing at 75°C for 3 min and cooling down at room temperature for 15 min. After addition of a final concentration of 200 μ M ATP, 4 μ M T4 DNA ligase and 0.2 unit/ μ L of SUPERase-In™ RNase inhibitor (Thermo Fisher Scientific), the reaction was incubated at 30°C for 3.5 to 16 hours.^{54–56}

The ligation efficiency was determined by primer extension analysis using a 6FAM labeled primer that anneals to nucleotides 114 to 95 for the 10-mer ribooligonucleotide ligation⁴¹ (5'-6FAM-GCC CTA GCT TAT GAC TTC GC-3', IDT) or nucleotides 28 to 1 for the 20-mer ribooligonucleotide ligation (5'-6FAM-CTG CAG CGA TTA CCC GAA CGT TAA GAA C-3'). The 6FAM-primer (9 pmol) was annealed to P RNA (27 pmol) in 50 mM Tris-HCl (pH 8.3), 75 mM NaCl and 10 mM DTT by denaturing for 3 min at 95°C followed by 15 min incubation at 37°C. A subsequent extension step took place at 42°C for 30 min after the addition of 10 mM MgCl₂, 0.8 mM dNTPs, and 2.5 unit/ μ L MuLV Reverse Transcriptase (Applied Biosystems). Reactions were visualized on a 6% denaturing gel, scanned using a Typhoon Phosphorimager (detection limit was determined to be 5 fmol of fluorescein) and quantified by ImageQuant or ImageJ software.

Determination of pre-tRNA dissociation constant by spin column

The affinity of ³²P 5' end labeled pre-tRNA^{Asp} with a 5-nt leader for RNase P holoenzyme was measured as described previously.^{57,58} The recombinant P protein was dialyzed against a binding buffer containing 50 mM Tris/MES, pH 6, ~380 mM KCl, and 5–10 mM CaCl₂ at 4°C. P RNA was folded by denaturation in water at 95°C for 3 min followed by 30 min incubation at 37°C before addition of the desired buffer. The P RNA was then buffer exchanged with the same buffer by Amicon centrifugation filters (MWCO 10K, Millipore) for at least three iterations. RNase P holoenzyme was reconstituted by mixing P RNA and P protein at a 1:1 ratio. RNase P holoenzyme (0–400 nM) was incubated with ³²P 5' end labeled pre-tRNA^{Asp} (<< 1 nM) for 5 min at 37°C. The holoenzyme-substrate mixture was loaded onto a pre-packed G-75 Sephadex (GE health) spin column (Corning Life Sciences).

The unbound substrate was separated from the mixture by centrifugation for 15 seconds at 6000 rpm. The amount (cpm) of bound and unbound substrate was measured by scintillation counting. Because the spin-column procedure does not completely separate free and bound ligand, the fraction of bound ligand was determined as $\text{fraction}_{\text{bound}} = (\text{fraction}_{\text{(cpm-bound)}} - \text{fraction}_{\text{(cpm-bound with no enzyme)}}) / (\text{fraction}_{\text{(cpm bound at endpoint)}} - \text{fraction}_{\text{(cpm bound with no enzyme)}})$.³⁴ A single binding isotherm (Equation 1) was fit to the data of fraction bound as a function of RNase P holoenzyme concentration to obtain the dissociation constant, $K_{d, \text{app}}$ at different Ca^{2+} concentrations (Figure S2). The fraction bound values were obtained from two to four independent experiments and errors are calculated as standard deviations.

$$\text{Fraction}_{\text{bound}} = \frac{[ES] + [ES^*]}{[S_{\text{total}}]} = \frac{1}{1 + \frac{K_{d, \text{app}}}{[E_{\text{total}}]}} \quad \text{Equation 1}$$

Single-turnover kinetic experiments

Single-turnover experiments were performed at saturating concentrations of divalent metal ions (20 mM MgCl_2 , 50 mM Tris/MES with varying pH, ~200 mM KCl for constant ionic strength at different pH, and 20 mM DTT at 37°C). Saturating concentrations of RNase P (360 to 500 nM) and limiting Fl-pre-tRNA^{Asp} substrate (10 nM) were used to investigate the catalytic activity of the variants. For metal rescue experiments, the single-turnover reactions were supplemented with 0.5 to 10 mM CdCl_2 (Figure 3) or MnCl_2 (Figure S4) with 20 mM MgCl_2 , 50 mM Tris/MES pH 5.1, 200 mM KCl at 37°C. DTT was not included in the metal-rescue experiments because precipitation was observed when DTT is incubated with Cd(II) and Mn(II). The single-turnover cleavage of Fl-pre-tRNA^{Asp} was measured by either: a fluorescence anisotropy assay (for $k_{\text{obs}} < 0.1 \text{ s}^{-1}$)⁵⁹ in a black 96-well microplate (Corning Incorporation, #3686) by monitoring the anisotropy change of fluorescein (filters with $\lambda_{\text{ex}} = 485 \pm 20 \text{ nm}$ and $\lambda_{\text{em}} = 535 \pm 25 \text{ nm}$) using a TECAN plate-reader (Infinite F500, G factor = 0.93);⁵⁹ or by quenching the reaction by an equal volume of EDTA quenching solution [10 M urea, 200 mM EDTA (pH 8.0), 0.05% bromophenol blue, and 0.05% xylene cyanol]⁵² at short time points ($< 5 \text{ sec}$ for $k_{\text{obs}} > 0.1 \text{ s}^{-1}$) using a mechanical rapid quench apparatus (Model RQF-3, Kintek Corporation) and separating products from substrates using a gel-based assay.⁶⁰

For the RNase P holoenzyme catalyzed single-turnover reactions measured by FA assay, the total fluorescence intensity decreased from the initial substrate-RNase P complex (F^0) to cleaved product (F^∞), and the extent of this decrease varies with pH. Therefore, an enhancement factor g , defined as described previously,⁶¹ was used to correct for the total fluorescence change when converting the anisotropy time course to a fraction cleaved (Y) trace using Equation 2. The enhancement factor is defined by $g = F^0/F^\infty$. The r_0 and r_∞ are the anisotropy values of the RNase P holoenzyme-bound substrate and cleaved product, respectively, measured for each experiment. All data analyses were performed using

GraphPad Prism 5.03 or 6.0 (GraphPad Software). Each experiment contains 2 to 5 independent measurements (error bars are showing standard deviations).

$$Y = 1 \frac{r - r_{\infty}}{r_0 - r_{\infty} + (g - 1)(r_0 - r)} \quad \text{Equation 2}$$

The single-turnover cleavage rate constant (k_{obs}) was then calculated by fitting a single exponential model (Equation 3) to the fraction cleaved as a function of time.

$$Y = Y_{\infty}(1 - e^{-k_{\text{obs}}t}) \quad \text{Equation 3}$$

The pKa values and pH-independent rate constants for reactions catalyzed by RNase P holoenzyme were calculated by fitting a single ionization model (Equation 4) to the pH profile (Figure 2A and B):

$$\text{pH} = \frac{k_{\text{max}}}{1 + 10^{\frac{(\text{pK}_a - \text{pH})}{n}}} \quad \text{Equation 4}$$

Fluorescence stopped-flow kinetic experiments

Stopped-flow experiments were performed on a KinTek model SF-2001 stopped-flow equipped with either a 75W or a 100W Xenon arc lamp in two-syringe mode, mixing 10 nM Fl-pre-tRNA^{Asp} with 360 nM RNase P holoenzyme. Fluorescein was excited at 488 nm and emission was monitored at wavelengths > 510 nm using a cut-on filter (Corion). Sum of exponentials (Equation 5) was fit to the transient kinetic time courses, $F(t)$, of three to eight independent determinations to obtain the fluorescence amplitude (A) and the observed rate constant, k_{obs} , for each exponential phase where $F(0)$ is the initial fluorescence intensity, and t , time:

$$F(t) = \sum A_n(e^{-k_{\text{obs}, N}t}) + F(0) \quad \text{Equation 5}$$

Data fitting was performed using GraphPad Prism 5.03 or 6.0 (GraphPad Software). The reported errors are the asymptotic standard errors. The traces for mixing substrate with RNase P holoenzyme in 20 mM Mg^{2+} were best fit by a three-exponential model (Figure S2) which is consistent with previous report⁶⁰ where $k_{\text{obs}2}$ corresponds to the conformational change step and $k_{\text{obs}3}$ corresponds to the cleavage step (Table S2). The errors are the standard error (SEM) from the best fit from the averaged trace of all data sets.

RESULTS AND DISCUSSION

Nucleotide substitutions of U51, G378 and G379 of RNase P

In contrast to previous site-directed mutagenesis studies,^{62,63} we chose nucleotide analog and abasic substitutions to probe proposed metal binding sites in P4 at a single-atom level with minimum disruption of the base-pairing geometry. The modifications of U51 in RNase P include 4-thiouridine (4SU), 4-deoxyuridine (4deOU), removal of the uracil base (rSpacer/abasic) and deletion of U51 (ΔU51) (Figure 1B). G378 and G379 were replaced with 2-aminopurine nucleoside (2AP), N7-deazaguanosine (7deNG), 6-thioguanosine (6SG) and abasic substitutions. Modifications were incorporated into P RNA by ligation of a 10-mer ribooligonucleotide to a circularly permuted P RNA (56P for U51)⁴¹ and a 20-mer ribooligonucleotide to a second circularly permuted P RNA (386P for G378 and G379). The truncated 56P⁴¹ and 386P RNA are inactive and do not interfere with measurements of the catalytic activity or substrate binding of ligated P RNA holoenzyme (data not shown). The wild-type-like circularly permuted P RNA constructs, 46P⁴¹ (for U51 variants, Figure 1A) and 366P RNA (for G378 and G379 variants), were used as controls and their single-turnover activity is comparable to that of wild-type (WT) (data not shown).

U51 substitutions decrease RNase P catalyzed cleavage activity

We measured the single-turnover cleavage of a 5' fluorescein labeled *B. subtilis* pre-tRNA^{Asp} catalyzed by WT and modified RNase P holoenzymes as a function of pH.⁵⁹ The rate-limiting step in the single-turnover rate constant (k_{obs}) for RNase P catalysis changes from phosphodiester bond cleavage at low pH to a conformational change step at high pH (Scheme 1, Figure 2).^{45,51} For WT RNase P, the k_{obs} value has a log-linear dependence on pH from pH 4.5 to 6.9, consistent with rate-limiting phosphodiester bond cleavage (Figures 2A&B).⁴⁵ Furthermore, isotope effects using ¹⁸O-water suggest a Mg(II)-bound hydroxide nucleophile in this reaction.⁶⁴ At high pH, the k_{obs} is no longer dependent on pH (Figures 2A&B), suggesting that the rate-limiting step switches to a conformational change step prior to substrate cleavage (transition from ES to ES* in Scheme 1).^{45,51} We measured single-turnover reactions in saturating Mg²⁺ (20 mM) and saturating RNase P at 37 °C. Under these conditions the pre-tRNA affinity of the modified RNase P enzymes is comparable to wild-type (Figure S2) and the k_{obs} value is independent of the enzyme concentration (data not shown). All of the modified RNase P enzymes catalyze formation of mature tRNA with no observable miscleavage (data not shown).

All of the U51 substitutions (4SU, 4deOU, abasic and ΔU51) decrease cleavage activity at low pH (rate-limiting cleavage). At pH 5.2, the k_{obs} values for these RNase P variants decrease by 19 to 23-fold at 37 °C (Figures 2A&C, Table S1). Consistent with this, fluorescence stopped-flow experiments⁴⁵ at 25 °C demonstrate that the cleavage step is rate-limiting at this pH for the U51-4SU variant (Figure S3, Table S2). The effect of the U51-4SU substitution on activity is smaller than the enormous activity decrease (300- to 10⁴-fold) observed for phosphorothioate modification of the proposed inner-sphere metal sites in the backbone phosphate of the P4 helix⁴⁰⁻⁴² and pre-tRNA substrate⁶⁵⁻⁶⁷. This small thio-effect observed for U51-4SU is likely due to the weaker affinity⁶⁸ of the carbonyl oxygen for Mg²⁺ compared to the negatively charged non-bridging phosphate oxygen.⁶⁹

Consistent with this, the U51-4SU substitution has the same effect on cleavage as removal of either the O4 moiety (4deOU) or the entire base (abasic) (Figure 2C, Table S1).

Metal-rescue of RNase P substitutions suggests inner-sphere metal ion coordination with O4 of U51

Some of the catalytic defects caused by substitution of a non-bridging phosphate oxygen with sulfur can be partially rescued by addition of metal ions that coordinate a sulfur ligand with higher affinity than Mg(II), such as Cd(II) and Mn(II),^{70,71} with relative rescue (k_{rel}) values of 2- to 200-fold, including RNase P.^{40–42,66,72–75} To further investigate the possibility that the O4 atom of U51 directly coordinates a Mg(II) ion, we carried out metal-rescue experiments using Cd(II) and Mn(II). As proposed, low concentrations of Cd²⁺ (up to 1 mM) in the presence of 20 mM Mg²⁺ activate cleavage catalyzed by U51-4SU RNase P by 2-fold (Figure 3B). In contrast, increasing concentrations of Cd²⁺ decrease the k_{obs} value for both the 46P control and U51-4deOU RNase P (Figures 3A&B). The relative rescue effect, $k_{\text{rel}} = (k_{\text{Cd}}/k_{\text{Mg}})^{4\text{SU}}/(k_{\text{Cd}}/k_{\text{Mg}})^{46\text{P}}$, shows that Cd²⁺ activates U51-4SU by up to $k_{\text{rel}} = 5$ -fold (Figure 3C). Additionally, the activity of U51-4SU RNase P is also enhanced upon addition of Mn²⁺ with a maximum $k_{\text{rel}} = 3$ -fold (Figure S4). No activation of the U51-4deOU or G379-6SG enzymes upon addition of Cd²⁺ (Figure 3C) is observed, suggesting that the Cd²⁺-rescue effect of U51-4SU is specific to the sulfur atom at position 4 (S4). In addition, the apparent dissociation constant of Cd²⁺ for rescue ($K_{1/2}^{\text{Cd}}$) increases with increasing Mg²⁺ concentrations (Figure 3D), indicating that Mg²⁺ competes with Cd²⁺ (Scheme S1). These data suggest that the S4 atom in U51-4SU makes a specific contact with Cd²⁺ and Mn²⁺ bound to the U51-4SU variant to activate RNase P catalysis. Therefore, these data argue that O4 of U51 forms an inner-sphere metal ion-binding site that activates catalytic activity, consistent with the two-metal-ion model proposed by the ternary complex crystal structure (Figure 4A).¹⁵

The U51 bulge contributes to the conformational change step

In contrast to the decrease on the cleavage rate constant, substitutions at U51 have little effect on the pH independent k_{max} where a conformational change step is rate limiting (Figure 2D).⁴⁵ The k_{max} value of U51-4SU (1.7 s⁻¹) is comparable the WT value (1.6 s⁻¹). Stopped-flow experiments confirm that the rate constant of the conformational change step of U51- 4SU RNase P is comparable to that of WT (Figure S3). Other substitutions lead to modest decreases in the conformational change step: U51-4deOU (1.0 s⁻¹), U51-abasic (0.8 s⁻¹) and U51 RNase P (0.4 s⁻¹). Similar decreases in the conformational change rate constant (2- to 5-fold) have previously been observed for alanine mutations in conserved arginine residues in P protein.⁷⁶ We speculate that the bulged U51 nucleotide contributes to the conformational change step during the RNase P-catalyzed reaction.

These data clarify the role of the universally conserved bulged U51 in the catalytic function of RNase P. Previously, site-directed mutations in *E. coli* P RNA that alter the bulged uridine lead to small to large effects on cleavage activity and/or substrate affinity.⁷⁷ In *T. maritima* RNase P, mutation of the bulged U to C decreases the catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$).⁶³ Our data provide direct biochemical evidence supporting inner-sphere metal coordination by the O4 carbonyl of the bulged uridine that enhances catalysis, as previously suggested by

structural studies (Figure 4A).¹⁵ Furthermore, this uridine may be involved in movements in the P4 helix during the conformational change in *B. subtilis* RNase P.

Effects of G378 and G379 substitutions suggest outer-sphere metal ion coordination and contribution to the conformational change step by G379

In contrast to U51, the modifications at G379 and G378 result in smaller decreases in activity and no observable metal-rescue of activity (Figure 2&3, Table S1). Although the G379-2AP (removal of the O6 oxygen) and G379-abasic variants decrease the cleavage rate constant (k_{obs} at pH 5.2) by 9- and 4-fold, respectively, (Figures 2B&C), both the cleavage rate constant and inhibition by Cd^{2+} of the G379-6SG (substitution of O6 by sulfur) is similar to that of the 46P control (Figure 3A&C). Therefore, O6 of G379 is required for efficient catalysis by RNase P but is unlikely to form an inner-sphere interaction with a metal ion. Furthermore, the G379-7deNG and G378-2AP variants have no effect on cleavage rates (Figures 2C&D, Table S1), suggesting that N7 of G379 or O6 of G378 do not interact with a catalytic metal ion. In summary, these data suggest that O6 of G379 may form a water-mediated (outer-sphere) interaction with a catalytic or co-catalytic metal ion that enhances cleavage (Figure 4B).

The values of the conformational change (k_{max}) for the G378-2AP (1.9 s^{-1}), G379-2AP (2.0 s^{-1}) and G379-7deNG (2.1 s^{-1}) variants are similar to WT (1.6 s^{-1}), demonstrating that the conformational change is not affected by these modifications (Figures 2B&D, Figure S3, Table S2). In contrast, the G379- abasic variant decreases k_{max} (0.5 s^{-1}) by 3-fold compared to WT RNase P, suggesting that the G379 guanine base is involved in the conformational change step during RNase P catalysis.

CONCLUSIONS

We used a combination of nucleotide substitution and kinetic studies to demonstrate that nucleobases in RNase P RNA interact with one or more catalytically important metal ion, likely via an inner-sphere interaction between Mg^{2+} and O4 of U51 (Figure 4A) and an outer-sphere interaction with O6 of G379 (Figure 4B). These nucleotides likely position the metal ions for efficient catalysis as well as stabilize the conformational transitions. This is the first direct biochemical evidence of inner-sphere metal ion coordination with a nucleobase in RNase P RNA that enhances cleavage.

This work and others^{21,22} have begun to demonstrate the biochemical importance of divalent metal ion coordination with nucleobase in catalytic RNA, which has been poorly understood compared to interactions with the phosphodiester backbone. Here, we provide a much-needed biochemical framework using single-atom and abasic substitutions to examine in detail the biochemical functions of nucleobases in catalytic RNAs. In addition, in contrast to the deleterious effect of abasic modifications in a small ribozyme,⁷⁸ our data demonstrate that in large RNAs the relatively stable RNA abasic⁷⁹ modification provides a new strategy to probe functions of conserved nucleobases, analogous to the alanine scanning mutagenesis strategy used to study proteins.⁸⁰ Therefore, this work lays the foundation for exploring the thousands of inner-sphere $\text{Mg}(\text{II})$ interactions with RNA nucleobases²² and with the fast-growing list of functional RNA modifications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) Secondary structure showing the catalytic domain of the full-length 46P circularly permuted *B. subtilis* P RNA with nucleotides U51 and G379 in bold and marked by asterisks. (B) Chemical structures of the nucleotide analogs used in this study.

Figure 2.

Base substitutions at U51 and G379 affect cleavage (k_{obs}) and/or conformational change steps (k_{max}). (A)(B) pH profiles of single-turnover cleavage activity (k_{obs}). (C) The k_{obs} for RNase P holoenzyme at low pH (5.1 or 5.2). (D) The pH-independent rate constant (k_{max} at high pH) is calculated from the pH profile.

Figure 3.

Cd^{2+} rescues single-turnover cleavage activity of U51-4SU RNase P holoenzyme. (A)(B) The dependence of the RNase P holoenzyme cleavage rate constant, k_{obs} , on $[\text{Cd}^{2+}]$ in 20 mM Mg^{2+} at pH 5.1. (C) Relative rescue of modified RNase P where $k_{\text{rel}} = (k_{\text{Cd}}/k_{\text{Mg}})^{4\text{SU}} / (k_{\text{Cd}}/k_{\text{Mg}})^{46\text{P}}$. 46P: WT-like control for circular-permutation; k_{rel} ratio-of-ratio for comparing modified RNase P to 46P in the presence of the equivalent amount and composition of metal ions; k_{Cd} : k_{obs} at a given $[\text{Cd}^{2+}]$ in 20 mM Mg^{2+} ; k_{Mg} : k_{obs} at 20 mM Mg^{2+} . (D) The apparent affinity of Cd^{2+} ($K_{1/2}^{\text{Cd}}$) for the rescue of U51-4SU activity decreases with increasing $[\text{Mg}^{2+}]$.

Figure 4.

(A) Model for Mg(II) ion (M) binding sites in helix P4 of P RNA in the transition state of RNase P-catalyzed phosphodiester bond cleavage.¹⁵ (B) O6 of G379 interacts with a Mg²⁺ ion through a water-mediated outer-sphere interaction.

Scheme 1. A minimum kinetic mechanism of bacterial RNase P^a

^aE: RNase P holoenzyme; S: pre-tRNA substrate; ES: RNase P•pre-tRNA complex; ES*: RNase P•pre-tRNA complex after conformational change; L: 5' leader; P: tRNA product.⁴⁵