

## A General RNA-Capping Ribozyme Retains Stereochemistry during Cap Exchange

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**Abstract:** Numerous natural and artificial ribozymes have been shown to facilitate reactions that invert stereochemistry. Here, we demonstrate that an RNA-capping ribozyme retains stereochemistry at a phosphorus reaction center. The ribozyme synthesizes a broad range of 5′–5′ RNA caps by exchanging phosphate groups around the  $\alpha$ -phosphate found at the 5′ terminus of the ribozyme. A ribozyme prepared with an Rp adenosine(5′) $\alpha$ -thiotetraphosphate cap was found to exchange this cap for an Rp 4-thiouridine-(5′) $\alpha$ -thiotetraphosphate cap when incubated with 4-thiouridine triphosphate. The same Rp capped construct, when incubated with [ $\gamma$ - $^{32}$ P]-ATP, exchanged the unlabeled ATP for a radiolabeled one while maintaining the same stereoconfiguration. In contrast, ribozymes prepared with an Sp cap failed to react even in the presence of thiophilic metal ions such as manganese. The kinetics of capping was also unusual as compared to inverting ribozymes. When the ribozyme was prepared with a triphosphate, capping was found to follow Michaelis–Menten-type kinetics even though the rate of pyrophosphate release was completely independent of nucleotide substrate concentration. Interestingly, the rate of capping and hydrolysis, when summed, was found to be indistinguishable from the rate of pyrophosphate release, indicating that an early rate-limiting step precedes both capping and hydrolysis. Together the retention of stereochemistry and kinetics imply that capping utilizes two inverting chemical steps that are separated by the transient formation of a rate-limiting covalent intermediate. As all protein enzymes that mediate similar capping reactions utilize a covalent intermediate, chemical necessity may have strongly guided the evolution of both protein and RNA-capping catalysts.

Ever since the discovery that RNA is capable of catalysis, there has been a fascination with ribozyme mechanism and stereochemistry. Early in the history of RNA catalysis, the divalent metal-dependent *Tetrahymena* self-splicing intron, the hammerhead ribozyme, and the group II intron were shown, via the use of phosphothioate modifications, to invert stereochemistry during phosphodiester bond formation and cleavage reactions (Figure 1a).<sup>1–4</sup> The in-line attack model that was developed on the basis of these results has since been validated and extended to ribozymes that mediate chemistry without the direct catalytic participation of magnesium ions, most notably the hairpin ribozyme that was recently crystallized using a vanadate pentacoordinate transition state mimic.<sup>5,6</sup>

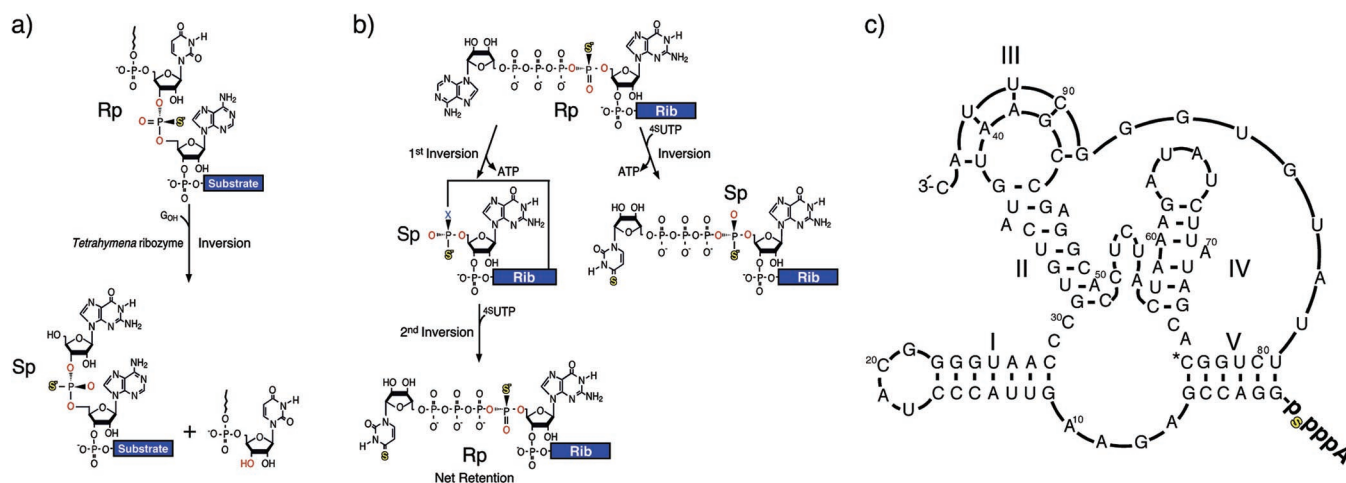
The perception that all ribozyme reactions involve single-step inverting reactions is deeply entrenched within the field of RNA biochemistry. It is useful, however, to consider other catalytic strategies that RNA can exploit to mediate reactions at phosphorus centers. Shortly after showing the catalytic ability of the *Tetrahymena* self-splicing intron, Zaug and Cech

demonstrated that it could be engineered to mimic both an alkaline phosphatase and a phosphoryl transferase, enzymes that utilize covalent intermediates.<sup>7</sup> However, questions concerning the stereochemistry and metal ion specificity of these constructs could not be addressed, because the reactive center within these ribozymes involves a phosphomonoester and not a phosphodiester. This made it practically impossible to track the reactions stereochemically by using atomic substitution of oxygen with sulfur. Thus, despite nearly two decades of research into RNA catalysis and the isolation of many exotic catalytic activities,<sup>8,9</sup> a ribozyme that utilizes a retaining mechanism has not been stereochemically characterized.

Capping a nucleic acid produces a 5′–5′ phosphate bridge between the nucleic acid and the capping nucleotide and has several important biological functions. Capping increases the lifetime of mRNA in eukaryotes, and enzymes such as T4 DNA and RNA ligase activate monophosphates to adenylates so as to facilitate the formation of phosphodiester linkages.<sup>10–12</sup> RNA capping uses GTP:RNA guanylyl transferase to transfer a GMP

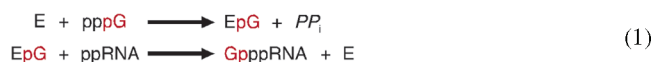
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**Figure 1.** Tracking capping stereochemistry using Rp or Sp thiophosphate-modified ribozymes. (a) Classically the endonuclease activity of the *Tetrahymena* ribozyme was shown to invert stereochemistry when an Rp thiophosphodiester was converted to an Sp form after attack by a guanosine nucleoside.<sup>1</sup> (b) The possible stereochemical outcomes of a cap exchange reaction where an Rp-capped ribozyme, (R)-Apppp<sub>5</sub>-Rib, is exchanged for a 4-thiouridine cap. (c) Proposed secondary structure of the c6.17 (shown activated with adenosine thiotetraphosphate cap) ribozyme.<sup>13</sup>

onto the 5'-diphosphate end of an mRNA to form a G(5')ppp-(5')-RNA cap. This enzyme-catalyzed reaction proceeds via a covalent enzyme-(lysyl-N)-GMP phosphoramidate intermediate that is the result of a nucleophilic attack by a lysine residue onto the  $\alpha$ -phosphate of GTP. The cap is formed when the  $\beta$ -phosphate of the mRNA 5'-diphosphate attacks the  $\alpha$ -phosphate of the GMP-enzyme intermediate, releasing it from the enzyme by displacement of the lysine residue (eq 1), with an overall retention of stereoconfiguration.<sup>10</sup> A similar mechanism is utilized by the DNA and RNA ligases to form a pyrophosphate bridge.



Here, we characterize the kinetics and reaction stereochemistry of a capping ribozyme called 6.17. The capping ribozyme was isolated from a high diversity random sequence RNA pool based on its ability to react with <sup>45</sup>UTP.<sup>13</sup> The 272 nt long construct was truncated into a fully functional 94 nt long cis construct shown in Figure 1c (called c6.17). A trans-acting construct (called t6.17), created by opening the loop of helix I, separates the ribozyme into two modules called A and B, with capping taking place at the 5'-end of module B.<sup>13</sup> A broad range of capping related chemistry takes place at the  $\alpha$ -phosphate of the ribozyme's 5' triphosphate (eq 2). This is in contrast to eukaryotic capping where the  $\alpha$ -phosphate of a GTP substrate serves as the reactive center. Capping proceeds with a wide variety of nucleotides and does not strongly depend on the identity of the base (Ade, Gua, Cyt, Ura, <sup>45</sup>Ura) or sugar (ribose, deoxyribose, and dideoxyribose), but requires at least one phosphate at the 5' position of the nucleotide involved in cap formation. The ribozyme also promotes a highly symmetric reaction, where the initial triphosphate is replaced with a tetraphosphate cap (Figure 1b, eq 3). This reaction allows the efficient exchange of one tetraphosphate cap for another. In the absence of nucleotide substrate, the ribozyme mediates a hydrolysis reaction where water attacks the  $\alpha$ -phosphate and inactivates the ribozyme. The ribozyme is optimally active in

the presence of magnesium ions and tolerates manganese and calcium ions with rates reduced by  $\sim 2$  times and  $\sim 7$  times, respectively.<sup>13</sup> In this report, we show that 6.17 performs capping with unusual rate-limiting kinetics that retains stereoconfiguration.



## Materials and Methods

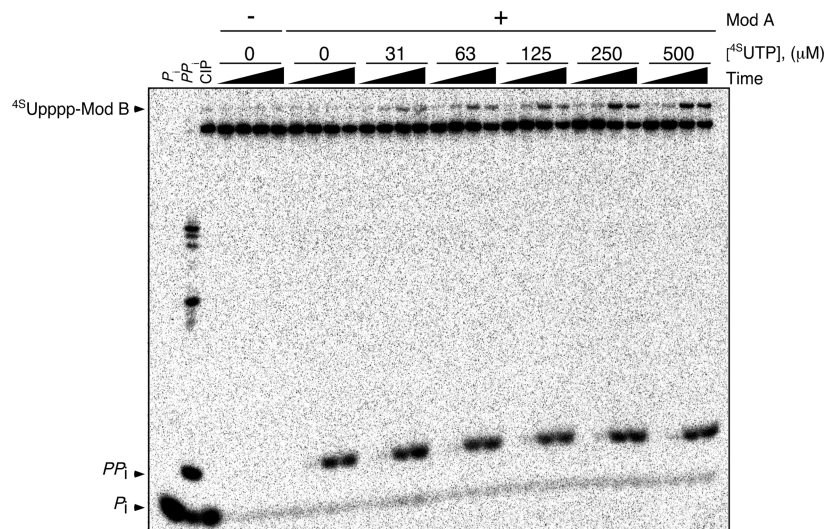
**Ribozyme Constructs.** RNA transcripts were made by in vitro T7 transcription [40 mM Tris-HCl pH 7.9, 2.5 mM spermidine, 26 mM MgCl<sub>2</sub>, 0.01% Triton X-100, 10 mM DTT, 8 mM GTP, 4 mM ATP, 4 mM CTP, 2 mM UTP, and 4 U/ $\mu$ L T7 RNA polymerase (T7 RNAP) incubated for 1 h at 37 °C].<sup>14</sup> The resulting transcripts were then gel purified using PAGE. The truncated cis-acting ribozyme (c6.17) has the following sequence (5'-gga ccg aga agu uac ccu acg ggg uaa ccc gug uca ugu aag ccg agg cac cuc uac uaa aga uau cuu aua gca cgg ucu uau gug ggc uua c) and has the GenBank accession number DQ371299. Module A of the trans construct has the following sequence (5'-ggg uaa ccc gug uca ugu aag ccg agg cac cuc uac uaa aga uau cuu aua gca cgg ucu uau gug ggc uua c), and module B has the following sequence (5'-gga ccg aga agu uac ccg cag agg aua cug acc).

**Pyrophosphate Release Kinetics.** Module B was transcribed in the presence of 2.5  $\mu$ Ci/ $\mu$ L [ $\gamma$ -<sup>32</sup>P]-GTP (3000 Ci/mmol, NEN) and gel purified. This module was then hybridized to Module A as previously described<sup>13</sup> in the standard incubation buffer (50 mM Tris-HCl pH 7.6, 150 mM KCl) supplemented with 20 mM MgCl<sub>2</sub>, and varying concentrations of <sup>45</sup>UTP at 22 °C. Control reactions were performed in the absence of module A or <sup>45</sup>UTP. The products were resolved on a 23% sequencing gel together with <sup>32</sup>P-inorganic phosphate and pyrophosphate markers. Inorganic phosphate was obtained by dephosphorylating [ $\gamma$ -<sup>32</sup>P]-ATP (NEN) using calf intestinal phosphatase (CIP, New England Biolabs); 50 mM Tris-HCl pH 7.9, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT supplemented with 1 U/ $\mu$ L of enzyme and incubating for 1 h at 37 °C. Pyrophosphate was made by saving an aliquot of a transcription reaction performed in the presence of [ $\gamma$ -<sup>32</sup>P]-GTP.

To correlate the capping rate with the pyrophosphate release rate, an [ $\alpha$ -<sup>32</sup>P]-UTP (NEN) body-labeled module B was mixed with [ $\gamma$ -<sup>32</sup>P]-GTP labeled module B with a specific activity ratio of 1:1 (by

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**Figure 2.** Rate of pyrophosphate release is independent of nucleotide substrate concentration. No pyrophosphate was released in the absence of module A, but when module A was present, pyrophosphate was released from module B with a rate that was independent of  $^{45}\text{UTP}$  concentration. During the  $^{45}\text{UTP}$  titration, the rate of capping increased and approached the rate of pyrophosphate release.

scintillation counting). This ratio was confirmed by phosphorimager quantification of a CIP-treated RNA sample of the mixture that was run on PAGE (found 51:49; Figure 2, CIP lane). The dual-labeled module B was then incubated with module A under varying concentrations of  $^{45}\text{UTP}$ . Time-points were taken at 0, 2, 24, and 48 h and were resolved using 23% *N*-acryloyl-aminophenyl-mercuric acid<sup>15</sup> (APM) gels.

**Capping Ribozyme Kinetics.** Module B was labeled on its 3' terminus with [ $^{32}\text{P}$ ]-pCp. This construct was then annealed to module A and incubated with varying concentrations of  $^{45}\text{UTP}$  in the standard incubation buffer supplemented with 20 mM  $\text{MgCl}_2$ . Aliquots were taken at 8, 16, 24, 48, 96, and 192 h and were separated on 10% APM gels. Fits were carried out as described in the text using the GraphPad Prism version 4.00 analysis package.

**Synthesis of Caps.** Guanosine  $\alpha$ -thiotriphosphate (GTP $\alpha\text{S}$ ) was purchased from TriLink Biotechnologies and separated into its Sp and Rp stereoisomers using reverse phase HPLC (ZORBAX XDB-C18 column on an Agilent 1100 series HPLC). After the column was equilibrated with 50 mM triethylammonium acetate pH 7.0 for 5 min at a flow rate of 1 mL/min, GTP $\alpha\text{S}$  was eluted using a linear gradient increasing by 1% acetonitrile per minute for 15 min. The Sp form eluted at  $\sim 7.3\%$  acetonitrile, while the Rp form eluted at  $\sim 8.1\%$ . The stereochemistry was assigned on the basis of two pieces of evidence: the Sp isomer elutes before the Rp, and that T7 RNAP is only able to utilize the Sp isomer during transcription.<sup>16</sup> Adenosine 5'-phosphorimidazolide (ImpA) and 4-thiouridine 5'-phosphorimidazolide (Imp $^{45}\text{U}$ ) were synthesized as previously described.<sup>17</sup> The tetraphosphates caps [AppppG, (S)-Apppp $\alpha\text{S}$ G, (R)-Apppp $\alpha\text{S}$ G, (S)- $^{45}\text{UppppG}$ , and (R)- $^{45}\text{UppppG}$ ] were synthesized by incubating the appropriate NTP at 50 mM with 200 mM ImpA or Imp $^{45}\text{U}$  in 50 mM HEPES pH 7.4 in the presence of 50 mM  $\text{MgCl}_2$  at 55  $^\circ\text{C}$  for 4 h. The reaction was stopped by quenching the magnesium with excess EDTA, and caps were purified using the HPLC conditions described earlier. Peaks that occurred as a result of the reaction were collected. The identity of the caps was confirmed by its UV-absorbance and mass spectrometry. The caps were dried using a speed-vac and resuspended in water.

**Synthesis of Modified Ribozymes and Capping/Exchange Reactions – APM Analysis.** Capped ribozymes were made by in vitro T7 RNAP transcription. GTP concentration was reduced to 1 mM to

encourage initiation with the dinucleotide caps, which were included at 1 mM in the reaction. The resulting RNAs were then gel purified using PAGE. Ribozymes capped with  $^{45}\text{U}$  [(S)- $^{45}\text{UppppG}$ -Rib and (R)- $^{45}\text{UppppG}$ -Rib] were further purified using an APM gel and used as stereochemical markers. Capping or exchange reactions were carried out by incubating [ $\alpha$ - $^{32}\text{P}$ ]-UTP internally labeled ribozymes in the standard incubation buffer in the presence of 10 mM  $\text{MgCl}_2$ , 5 mM  $\text{MnCl}_2$ , and 1 mM  $^{45}\text{UTP}$ . Time-points were taken at 0, 3, 24, and 48 h and resolved using APM gels. RNAs were treated with CIP to remove the triphosphate from ribozymes that initiated with GTP rather than dinucleotide caps during the transcription reactions. Reactions with these CIP-treated ribozymes were conducted as before.

#### HPLC Confirmation of the Capping Products Stereochemistry.

Unlabeled ppp-Rib and (R)-Apppp $\alpha\text{S}$ -Rib that had been CIP-treated after transcription were reacted at 2  $\mu\text{M}$  concentration with 5  $\mu\text{Ci}/\mu\text{L}$  of [ $\gamma$ - $^{32}\text{P}$ ]-ATP in the same buffer used for the APM analysis. Aliquots were taken at 0, 0.5, 3, and 20 h and gel purified. These samples were then digested with 100 U/ $\mu\text{L}$  T1 (Fermentas) and 1 U/ $\mu\text{L}$  T2 (Fermentas) at 37  $^\circ\text{C}$  for 15 min, followed by treatment with 1 U/ $\mu\text{L}$  CIP for 1 h to remove the 3'-monophosphates resulting from the digestion. Unlabeled AppppG, (S)-Apppp $\alpha\text{S}$ G, and (R)-Apppp $\alpha\text{S}$ G standards (see cap synthesis section) were added to these tubes and fractionated by HPLC, using the same conditions mentioned earlier. Fractions were collected every 2 min, between 0 and 12.5 min, every 6 s, between 12.5 and 15.1 min, and every 2 min, between 15.1 and 20 min. The fractions were then counted on a Beckman LS600SC scintillation counter for 9 min in a total fluid volume of 7 mL (2 mL of aqueous sample mixed with 5 mL of Formula-989 scintillation fluid).

## Results

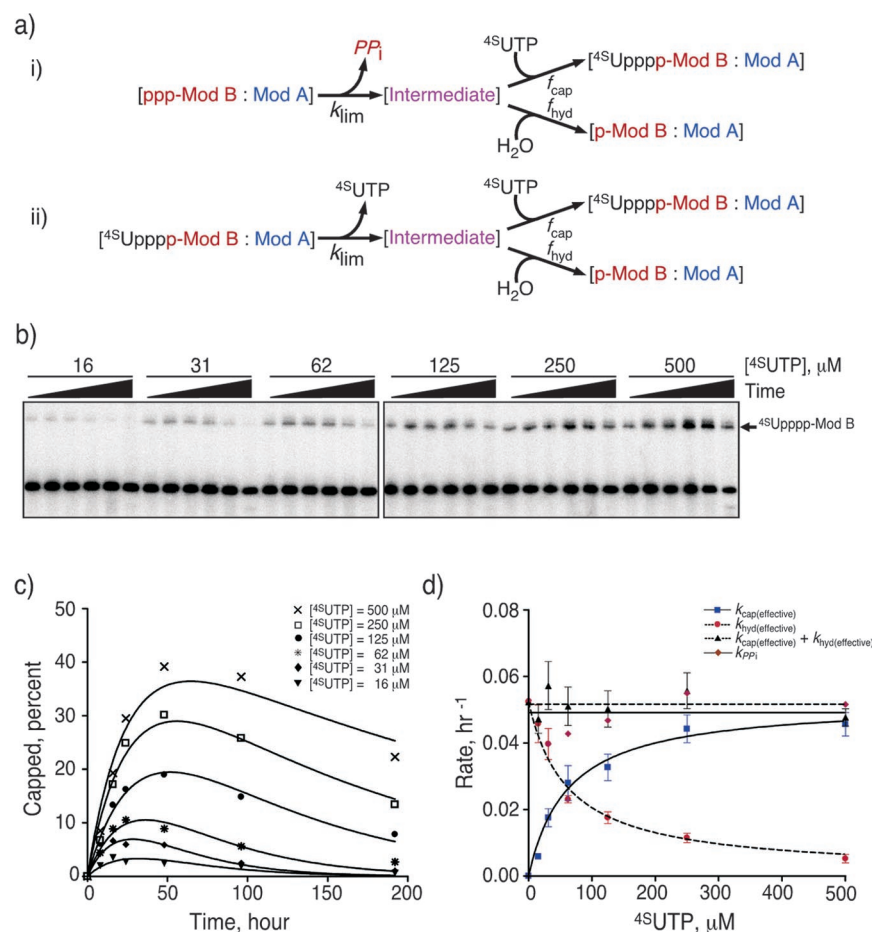
**A Rate-Limiting Step Precedes Capping.** A simple kinetic model requiring an early rate-limiting kinetic intermediate fit a broad range of data. This model was initially suggested by the finding that the rate of pyrophosphate release was fixed and independent of the overall rate of capping (Figure 2). The model assumes that both the triphosphate and the capped forms of module B substrates have equivalent kinetics and react to form an early rate-limiting kinetic intermediate (Figure 3a). This intermediate was found to partition into either a capped ( $f_{\text{cap}}$ ) or an inert hydrolyzed product ( $f_{\text{hyd}} = 1 - f_{\text{cap}}$ ). Consistent with this model, a UTP capped module B was able to be recapped with  $^{45}\text{UTP}$  with the rate of this exchange increasing with  $^{45}\text{UTP}$

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**Figure 3.** The kinetics of capping using  $^{45}UTP$  as a substrate. (a) A kinetic model for the trans form of the ribozyme. (b) APM-polyacrylamide gel showing one of five replicates used to determine capping kinetics, the lower band on the gel being unreacted or hydrolyzed module B. (c) The  $^{45}UTP$ -dependent temporal fit to the data in (b). (d) The capping rates fit well to a simple Michaelis–Menten-type curve, while hydrolysis rates agreed well with the model in (a).

concentration. At the same time, the rate of hydrolysis was observed to fall in direct correspondence.<sup>13</sup> Together the two reaction schemes shown in Figure 3a define a differential equation that predicts the amount of  $^{45}UTP$  capped module B as a function of time:

$$F = f e^{-k_{hyd(effective)}t} - e^{-(k_{cap(effective)} + k_{hyd(effective)})t}$$

with  $f$  being the maximum fraction able to react (and was set to 0.5 based on an analysis of pyrophosphate release shown in Figure 2),  $k_{cap(effective)} = f_{cap}k_{lim}$  and  $k_{hyd(effective)} = f_{hyd}k_{lim}$ . A representative set of time-dependent data and the resulting fits are shown in Figure 3b and c. The effective capping and hydrolysis rates were then averaged from five independent data sets and plotted as a function of  $^{45}UTP$  concentration (Figure 3d). The effective rate of capping was found to fit well to a Michaelis–Menten-type equation where  $k_{cap(effective)} = k_{lim}[^{45}UTP]/(K_m + [^{45}UTP])$ . The effective hydrolysis rate was allowed one additional degree of freedom and fit to  $k_{hyd(effective)} = k_{lim}[K_m]/(K_m + [^{45}UTP]) + k_{hyd(min)}$  (Figure 3d). A nonzero value for  $k_{hyd(min)}$  would imply that  $f_{hyd}$  did not equal  $1 - f_{cap}$  and contradict our model. The simultaneous fits agreed very well with the data with a  $k_{lim}$  of  $0.052 \pm 0.002 \text{ h}^{-1}$ , a  $K_m$  of  $62 \pm 16 \mu M$ , and a  $k_{hyd(min)}$  of  $0.001 \pm 0.002 \text{ h}^{-1}$ . As expected from this model, the value of  $k_{cap(effective)} + k_{hyd(effective)}$  nearly equaled the rate of pyrophosphate release determined independently at

the same  $^{45}UTP$  concentration (Figure 3d,  $\blacktriangle$  and  $\blacklozenge$ , respectively). Indeed, the average rate of pyrophosphate release was  $0.050 \pm 0.003 \text{ h}^{-1}$ , equaling  $k_{lim}$  to within error. This simple kinetic model explains a broad range of capping data and has the unusual feature that the rate of pyrophosphate release is invariant and not a function of substrate concentration as is typical for most ribozymes that liberate pyrophosphate during catalysis.

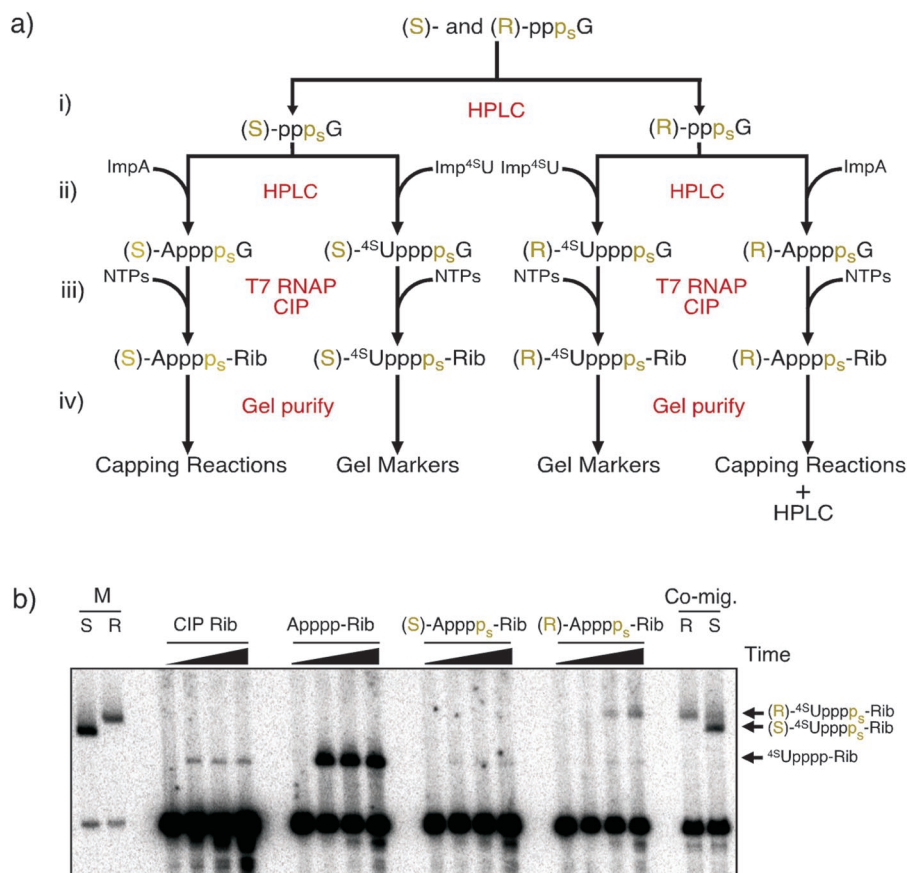
The kinetics of t6.17 bear an uncanny resemblance to those of a calcium-dependent capping ribozyme called Iso6.<sup>18</sup> Both ribozymes share the property that the rate of pyrophosphate release is independent of substrate concentration (Figures 2 and 3 for t6.17).<sup>19</sup> Further, decreasing the phosphate chain length of the nucleotide substrate to a monophosphate has a marked effect on binding affinity for both ribozymes, but leaves the saturated rate of capping unchanged for both ribozymes.<sup>13,19</sup> Additionally, both tolerate a broad range of nucleotide substrates and have similar pH dependence<sup>13,18–21</sup> even though the ribozymes have different metal ion requirements and secondary structures.<sup>13,20</sup> To our knowledge, Iso6 and 6.17 are the only ribozymes that share such a detailed range of properties. The simplest interpretation of these similarities is that a covalent

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**Figure 4.** Capping proceeds with retention at the reactive (5′) α-phosphate center – APM analysis. (a) Synthesis flowchart leading to gel-purified Rp- or Sp-capped ribozymes possessing either adenosine or 4-thiouridine caps. (b) Demonstration that ribozyme c6.17 forms caps that are retained relative to the initial substrate stereochemistry.

intermediate is formed by both ribozymes during capping. Although a lariat-type structure of the form suggested in Figure 1b was not detected for either ribozyme despite careful gel-based analysis, its presence can be inferred by probing the stereochemistry of the reaction. A covalent intermediate would require two sequential nucleophilic attacks, with each inverting the stereochemistry of the α-phosphate found on the 5′ terminus of the ribozyme, resulting in a net retention of stereoconfiguration, while a single-step reaction would be expected to invert stereochemistry.

**Ribozyme c6.17 Performs Capping with Net Retention of Stereoconfiguration around the α-Phosphate.** Stereochemistry was monitored by rendering the reactive 5′ α-phosphorus of each ribozyme chiral by substituting either of its nonbridging oxygens with a sulfur. Two sets of dinucleotide cap stereoisomers were synthesized (Figure 4a). The first, Sp and Rp adenosine(5′)α-thiotetraphospho-(5′)guanosine [(S)- and (R)-Apppp<sub>5</sub>G, respectively], were used to prepare ribozymes with a stereochemically modified cap and were also used as absolute references. The second set, Sp and Rp 4-thiouridine(5′)α-thiotetraphospho-(5′)guanosine [(S)- and (R)-<sup>45</sup>Upppp<sub>5</sub>G, respectively], were used to generate stereochemical gel mobility markers. Caps are typically incorporated during the initiation of transcription by T7 RNA polymerase<sup>22</sup> and, as we show, allow the synthesis of ribozyme transcripts containing either Sp or Rp (5′) α-thiotetraphosphate caps. Initiation with these

caps rather than GαSTP prevented the otherwise unavoidable incorporation of Sp GαSMP into the backbone of the RNA transcripts. The caps were synthesized by first purifying Sp and Rp GαSTP [(S)-ppp<sub>5</sub>G and (R)-ppp<sub>5</sub>G respectively], and their absolute stereochemistry was confirmed by their enzymatic utilization and relative HPLC retention times.<sup>16</sup> Each stereoisomer was then reacted with adenosine 5′-phosphorimidazole (ImpA) or 4-thiouridine 5′-phosphorimidazole (Imp<sup>45</sup>U)<sup>17</sup> so as to produce Sp or Rp dinucleotide α-thiotetraphosphate caps that were subsequently HPLC purified (Figure 4a). Mass spectrometry and UV spectra together with the relative HPLC retention times of each pair confirmed the expected products. Transcription in the presence of these caps resulted in their incorporation on the 5′ termini of RNA transcripts. (S)- and (R)-<sup>45</sup>Upppp<sub>5</sub>-RNA shifted significantly higher on an APM gel (as a result of the gels thiol–mercury-dependent interactions) than <sup>45</sup>Upppp-RNA, consistent with the two sulfur groups present in the (S)- and (R)-<sup>45</sup>Upppp<sub>5</sub>-RNA constructs. Conveniently, the (R)-<sup>45</sup>Upppp<sub>5</sub>-RNA shifted higher than (S)-<sup>45</sup>Upppp<sub>5</sub>-RNA, making it possible to monitor capping ribozyme stereochemistry by gel mobility (Figure 4b, left two lanes, indicated by M/(S and R)).

Only the (R)-Apppp<sub>5</sub>-Rib(c6.17) ribozyme construct was found to perform cap exchange, even though the (S)- and (R)-Apppp<sub>5</sub>-Rib constructs for Iso6 and c6.17 were tested with a broad range of thiophilic metal ions (Mn, Cd, Pb, Zn, Ni, Fe, and Cu). (S)- and (R)-Apppp<sub>5</sub>-Rib and an Apppp-Rib control were treated with calf intestinal phosphatase (CIP) after

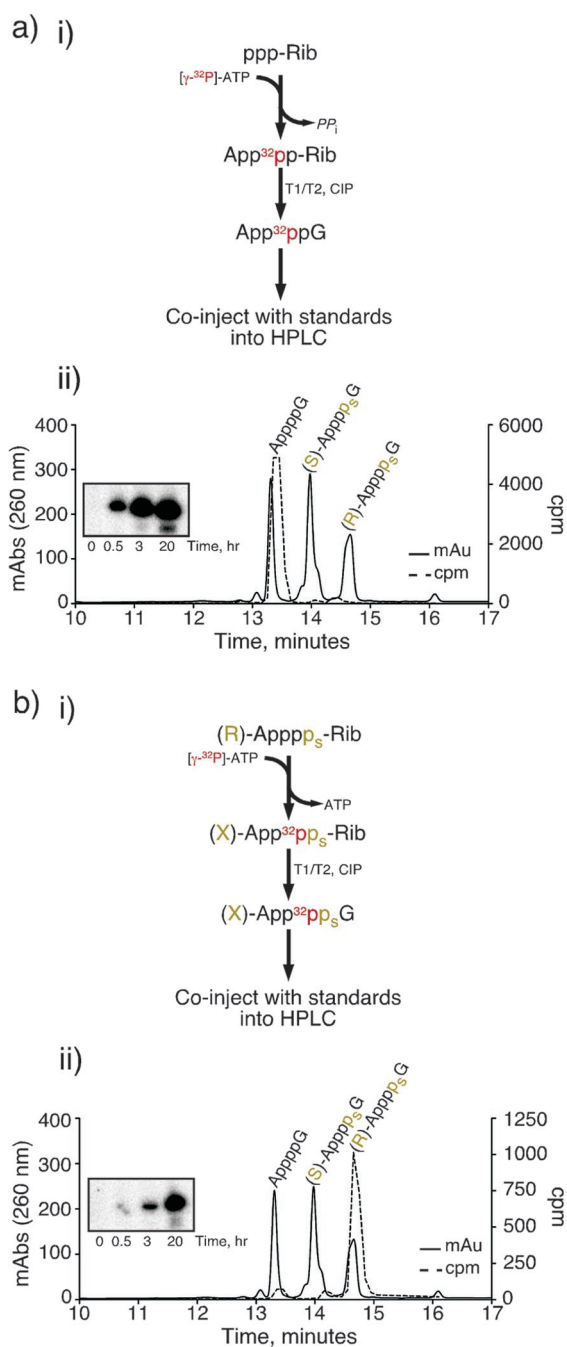
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transcription and gel purification so as to remove the 5' triphosphates that inevitably result from initiation with GTP rather than the desired dinucleotide cap. CIP was highly effective in removing this undesired 5' triphosphate, resulting in the nearly complete suppression of  $^{45}\text{S}$ UTP capping in a control reaction (compare Figure 4b CIP Rib lanes to Figure S1 ppp-Rib lanes). Apppp-Rib activity was preserved as expected, given the inability of CIP to dephosphorylate a cap structure (Figure 4b, Apppp-Rib lanes; Figure S1). When (S)-Apppp<sub>s</sub>-Rib was incubated with  $^{45}\text{S}$ UTP, no capping was observed under all tested conditions (Figure 4b, (S)-Apppp<sub>s</sub>-Rib). However, the (R)-Apppp<sub>s</sub>-Rib(c6.17) construct [Figure 4b, (R)-Apppp<sub>s</sub>-Rib] was active at approximately one-tenth the rate of the unmodified construct in optimal conditions using 10 mM magnesium and 5 mM manganese. Removing manganese from the reaction resulted in a modest 2-fold decrease in overall rate (data not shown). The reaction product, when mixed with a control (R)- $^{45}\text{S}$ Upppp<sub>s</sub>-Rib transcript, resulted in a single band, but two distinct bands were observed when it was mixed with the (S)- $^{45}\text{S}$ Upppp<sub>s</sub>-Rib marker (Figure 3b: Co-mig. lanes R and S, respectively). This strongly suggests that the (R)-Apppp<sub>s</sub>-Rib(c6.17) construct retained stereochemistry during the cap exchange reaction.

A second independent experiment was performed to confirm this finding. (S)- or (R)-Apppp<sub>s</sub>-Rib(c6.17) together with the equivalent Iso6 constructs were transcribed in the absence of a radiolabel, gel purified, and CIP treated to remove triphosphate as described (i.e., Figure 4a). As expected, when incubated with  $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ , only the (R)-Apppp<sub>s</sub>-Rib(c6.17) stereoisomer was able to exchange its cap for a radio-labeled nucleotide. As a control, ppp-Rib(c6.17) (transcribed in the absence of an initial cap) became capped with  $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$  (Figure 5a.ii and b.ii gel insets, Figure S2a). The resulting radio-labeled materials from the control and the (R)-Apppp<sub>s</sub>-Rib(c6.17) incubations were carefully gel purified and digested to completion with a mixture of T1 and T2. CIP was then added to remove any external phosphates (Figure S2b). Again, as expected, digestion of the control reaction (Figure 5a.i) left a radio-labeled cap with the same HPLC retention time as the AppppG standard (Figure 5a.ii, the solid line shows the UV trace of the dinucleotide cap standards, while the dotted lines indicate the eluted radioactivity in cpm). Digestion and CIP treatment of the (R)-Apppp<sub>s</sub>-Rib(c6.17) product yielded a radio-labeled cap that coeluted with the (R)-Apppp<sub>s</sub>G dinucleotide standard, which was used initially to make the construct (Figure 5b.ii). Thus, in two separate experiments, one involving exchange with  $^{45}\text{S}$ UTP, and the second with  $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ , the (R)-Apppp<sub>s</sub>-Rib(c6.17) construct was shown to retain stereochemistry.

## Discussion

The retaining mechanism of capping has an unusual metal ion dependence. Ribozymes that perform inverting phosphodiester chemistry fall into two general categories, depending on whether divalent metal ions directly participate in catalysis. The metal-dependent *Tetrahymena*, hammerhead, and group II intron ribozymes tolerate substitution by only one thiophosphate stereoisomer and not the other,<sup>1,3,4</sup> and are inactive in the presence of cobalt hexammine.<sup>13,23</sup> The inactive stereoisomer, in the case of the hammerhead, can be rescued by the addition



**Figure 5.** (R)-Apppp<sub>s</sub>-Rib(c6.17) produces a radio-labeled dinucleotide cap when reacted with  $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$  that has the same HPLC retention time as an (R)-Apppp<sub>s</sub>G cap standard. (a.i) Protocol used to manufacture a control radio-labeled dinucleotide cap synthesized by a triphosphate-containing ribozyme. (a.ii) HPLC chromatogram showing that the radio-labeled cap elutes with the AppppG standard as expected. (b.i) The same protocol was used for (R)-Apppp<sub>s</sub>-Rib(c6.17). (b.ii) This time the radio-labeled cap was found to elute at the same time as the (R)-Apppp<sub>s</sub>-G standard.

of soft metals such as manganese and cadmium.<sup>24</sup> The hairpin ribozyme in contrast is active in the presence of cobalt hexammine<sup>5</sup> and mediates reactions with both Rp and Sp thiophosphates. The finding that our retaining capping ribozyme reacts with only an Rp  $\alpha$ -thiophosphate substitution and not an Sp substitution is similar to the stereochemical signatures of the metal-dependent *Tetrahymena*, hammerhead, and group II

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intron ribozymes, which invert stereochemistry. However, while our retaining ribozyme fails to react in the presence of cobalt hexammine, we find no evidence that soft metals rescue the (S)-Apppp<sub>s</sub>-Rib(c6.17) construct. These observations indicate that the ribozyme makes critical contacts with the Sp oxygen of the  $\alpha$ -phosphate during the course of chemistry and hint that a divalent metal ion may be directly involved in capping chemistry by coordinating the *pro*-S oxygen on the  $\alpha$ -phosphate of the ribozyme during catalysis.

The retention of stereochemistry strongly suggests that the c6.17 ribozyme utilizes a two-step mechanism. Each step would invert stereochemistry and be separated by a transient covalent intermediate. Entirely consistent with this mechanism is the unusual finding that pyrophosphate release is independent of nucleotide substrate concentration during capping. A rate-limiting chemical step can easily be interpreted by a two-step model if the first step is slow and the second step is rapid and partitions the reactive covalent intermediate into either a cap or a hydrolysis product (Figures 1b and 2). While we could not isolate this covalent intermediate, its steady-state concentration would be expected to be low given the available kinetics. We are therefore confident, given the two independent data sets, that the retention of stereochemistry observed for c6.17 results from the formation of a transient covalent intermediate during capping.

Formally, this covalent intermediate could involve a ribose 2'-hydroxyl forming a phosphodiester intermediate, or a nitrogen from a base generating a phosphoramidate intermediate. Resolving these two possibilities is a difficult task, which we are currently exploring. A candidate nucleotide responsible for forming the intermediate is A<sub>75</sub> in the *cis*-acting construct (Figure 1c). Mutating this residue to a G completely inactivates the ribozyme, while substituting it for a thiophosphate-modified deoxyadenosine does not significantly affect catalytic rate (data not shown). Moreover, this residue is adjacent to the location of a quantitative cross-link (indicated by an asterisk in Figure 1c) between the 4-thiouridine cap product and the equivalent residue in the *trans* form of the ribozyme.<sup>13</sup> The inhibition of capping observed when this residue is mutated suggests that a nitrogen in the base is involved in forming the capping intermediate and not a hydroxyl from the backbone sugar. If true, exciting mechanistic parallels between 6.17 and a capping DNzyme selected by Yi and Breaker<sup>25</sup> should exist. This DNzyme is likely to utilize a phosphoramidate type intermedi-

ate, although the detailed mechanism for this DNzyme is currently unknown. The formation of a reactive phosphoramidate intermediate during capping is consistent with a short-lived kinetic intermediate and would be analogous to the utilization of the  $\epsilon$ -N of lysine residues seen in protein-catalyzed capping reactions.<sup>26</sup>

Why might capping utilize a retaining mechanism? Capping involves two charged phosphate groups that must be exchanged in a highly symmetrical reaction. This differs fundamentally from previously stereochemically characterized ribozymes that promote reactions at a phosphorus center and that use hydroxyl groups as nucleophiles or nucleofuges. Performing capping in a single step would require the simultaneous stabilization of two highly negatively charged phosphate groups that are in turn proximal to a negatively charged reactive center, a strategy unlikely to be optimal for either a protein or an RNA-based catalyst. A two-step reaction involving the sequential displacement of a charged nucleofuge followed by the attack of a nearly identical nucleophile requires a more complicated reaction landscape, but at the same time the two transition states encountered during the reaction would be expected to have dramatically lower activation energies than a single-step reaction. Equally important, by exploiting a two-step mechanism, where the reaction is symmetrical with respect to the reaction coordinate, only one substrate-binding site, which recognizes both the initial nucleofuge and the incoming nucleophile, would be required for catalysis. Thus, in contrast to the vast majority of biological reactions that invert stereochemistry, the evolution of capping biochemistry may have been driven inexorably to select a retaining mechanism irrespective of whether nucleic acid or protein mediates the reaction.

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**Supporting Information Available:** Figures S1 and S2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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