Oligoribonucleotides

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A Mini-Twister Variant and Impact of Residues/Cations on the Phosphodiester Cleavage of this Ribozyme Class

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Abstract: *Nucleolytic ribozymes catalyze site-specific cleavage of their phosphodiester backbones. A minimal version of the twister ribozyme is reported that lacks the phylogenetically conserved stem P1 while retaining wild-type activity. Atomic mutagenesis revealed that nitrogen atoms N1 and N3 of the adenine-6 at the cleavage site are indispensable for cleavage. By NMR spectroscopy, a pK_a value of 5.1 was determined for a ^{13}C2-labeled adenine at this position in the twister ribozyme, which is significantly shifted compared to the pK_a of the same adenine in the substrate alone. This finding pinpoints at a potential role for adenine-6 in the catalytic mechanism besides the previously identified invariant guanine-48 and a Mg^{2+} ion, both of which are directly coordinated to the non-bridging oxygen atoms of the scissile phosphate; for the latter, additional evidence stems from the observation that Mn^{2+} or Cd^{2+} accelerated cleavage of phosphorothioate substrates. The relevance of this metal ion binding site is further emphasized by a new 2.6 Å X-ray structure of a 2′-OCH_3-U5 modified twister ribozyme.*

Small self-cleaving ribozymes are widely distributed in nature[1] and are essential for rolling-circle-based replication of satellite RNAs.[2,3] Among them, the hepatitis delta virus (HDV) ribozyme[4–8] employs a divalent cation in the active site for catalysis, while the remaining small self-cleaving ribozymes including hammerhead,[2,9,10] hairpin,[3,11–13] glmS,[14–16] and Varkud Satellite[17] employ principles of general acid–base and electrostatics for catalysis. Very recently, a new class of nucleolytic ribozymes (termed twister) has been discovered,[18] and soon thereafter, crystal structures were published that revealed a common double-pseudoknot overall architecture for the twister ribozyme but showed clear distinctions in residue and divalent cation alignments at the cleavage site.[19–21] While the *O. sativa* twister ribozyme was off-line orthogonally aligned with a fully base-paired stem P1,[19,20] the *env22* twister ribozyme was in-line oriented at the cleavage step A6-U5, with a Mg^{2+} coordinated to the scissile phosphate. Furthermore, for the *env22* twister ribozyme, stem P1 formed only the two central base pairs (Figure 1) while the neighboring nucleotides U1 and U4 were instead engaged in

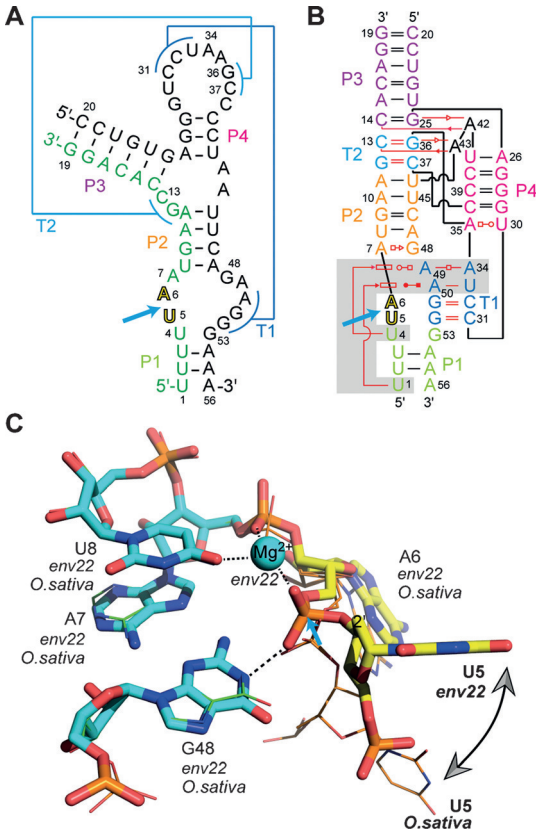

Figure 1. The *env22* twister ribozyme. Sequence in A) secondary structure presentation, and B) Leontis–Westhof presentation;[47,48] triplet formation is highlighted by a gray background. C) Overlay of X-ray structures PDB: 4OJI (*O. sativa* thin lines) and PDB: 4RGF (*env22* sticks) of the nucleosides at the cleavage step A6-U5 (yellow). Note that a rotation of the off-line oriented U5 (*O. sativa*) is required to take the in-line position of U5 (*env22*), indicated by arrow with gray heads. The C2′ of dU5 used for the crystal structure is labeled with 2′.

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stacked base triplet interactions (U4-A49-A34 and U1-A50-U33; Figure 1B).[21]

These contrasting observations were the starting point for the present investigation. A thorough comparison of the two structures (PDB: 4OJI for *O. sativa* and PDB: 4RGF for *env22*) revealed that the conserved adenosine (A6; *env22* numbering is used throughout) at the cleavage site adopts nearly identical conformations involving extensive hydrogen bonding networks and stacking interactions whereas the two consecutive (non-conserved) nucleosides upstream of the cleavage site (U5 and U4) are located in significantly distinct conformations in the two structures. These differences may, however, be interpreted as intrinsic conformational flexibility that is required for this ribozyme to function, with the two crystal structures representing snapshots of important conformations of the cleavage site. To this end, we speculated that stem P1 might not be as critical for cleavage activity as implied by phylogenetic analyses. We hypothesized that nucleosides U5 and U4 could attain even more conformational flexibility if they were not constrained by (transient) pairing of U1-U2-U3 within stem P1. Therefore, we systematically investigated truncated substrate strands in conjunction with the 37 nt ribozyme strand (Figure 2A). Strikingly, the twister ribozyme was able to cleave even a single nucleotide (U5) with an efficiency that was comparable to twister ribozymes that comprise an intact stem P1 (Figure 1A). Also, a minimal bimolecular twister variant that lacked U1-U2-U3-U4 and G53-A54-A55-A56 (Supporting Information, Figure S1), as well as a monomolecular, circularly permuted variant without stem P1 (Figure 2B; Supporting Information, Figure S1) showed highly efficient phosphodiester cleavage activity.

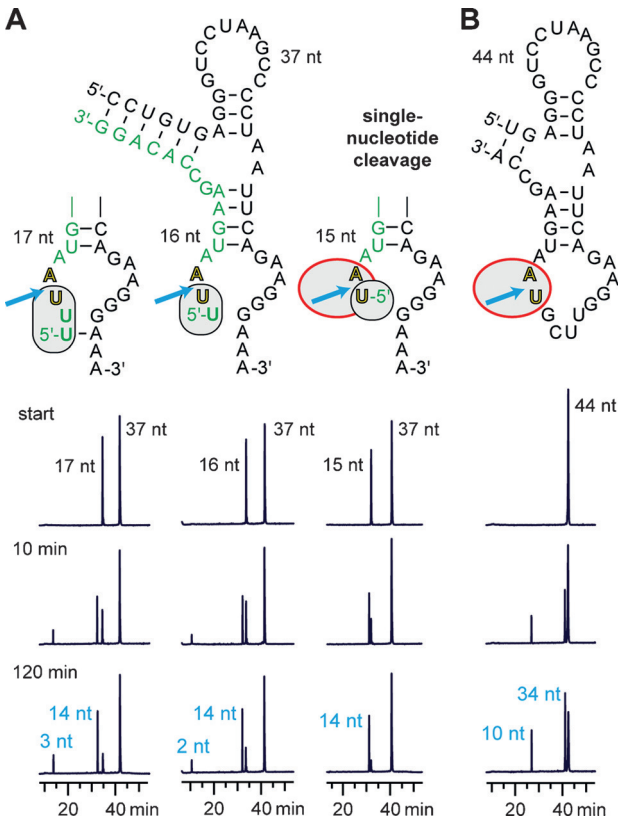

Figure 2. The twister ribozyme does not require the phylogenetically conserved stem P1 for efficient cleavage. A) HPLC cleavage assays of systematically truncated substrate RNAs, showing that even a single nucleotide (U5) is cleaved. B) A circularly permuted twister variant that lacks stem P1 self-cleaves efficiently. Conditions: 2 mM $MgCl_2$, 100 mM KCl, 30 mM HEPES, pH 7.5, 23 °C.

We furthermore determined the precise rate for single-nucleotide cleavage using a fluorescence-based, real-time cleavage assay (Supporting Information, Figure S2). The apparent rate k_{obs} of 0.88 ± 0.12 min^{-1} was in the same order as for substrates that can form stem P1 and that we reported previously (k_{obs} of 1.41 ± 0.16 min^{-1}).[21]

In this context, we mention that single nucleotide cleavage has been described for one other ribozyme, the HDV ribozyme.[22] We further point out that for the HDV ribozyme, a Mg^{2+} ion plays an essential role in catalysis of phosphodiester cleavage.[4,6,7,16]

Next, we examined the functional role of the invariant adenine (A6 *env22*) at the cleavage step. In both structures, this adenine is stacked on a non-canonical *trans*-Watson–Crick–Hoogsteen pair (U30 A35 *env22*) and its ribose 2′ hydroxy group establishes a hydrogen bond to N3 of A34. Even more importantly, hydrogen bonding of the exocyclic amino group to the non-bridging oxygen of the C31–C32 phosphate appears to be crucial (Figure 3A); additionally, this amino group is clamped by hydrogen bonding to the non-bridging oxygen of the neighboring C30–C31 phosphate (Figure 3A). We were therefore not surprised that shifting the exocyclic amino group from the 6 to the 2 position (by replacing adenine with 2-aminopurine) is deleterious (Figure 3B, middle left panel) most likely because proper positioning of A6 becomes impossible on losing these interactions. This interpretation is supported by the observation that cleavage activity can be restored with 2,6-diaminopurine (Figure 3B, middle right panel). Most interestingly, however was the finding that a variant that can properly interact with the C31–C32 phosphate but lacks N1 and N3 of the purine base (c1c3A) was not cleaved. Stimulated by these findings and the possible impact this adenine may have in acid–base catalysis, we synthesized a ^{13}C2-A6 labeled, non-cleavable ribozyme substrate (containing 2′-OCH_3-U5) to determine the pK_a of this adenine, by pH-dependent NMR spectroscopy experiments.[23] The pK_a values of A6 were determined by non-linear least-squares fits of the pH-dependent chemical-shift change of the ^{13}C2 resonance between pD 3.0 and 7.5 (Figure 4). The substrate strand alone displayed a pK_a of 3.7 ± 0.1, very well in accordance with reported pK_a values of unperturbed adenines in RNA.[24–28] However, when the substrate was annealed in the twister complex, the observed pH dependence indicated an acid–base equilibrium that was clearly shifted towards the neutral pH range, with a pK_a of 5.1 ± 0.1 (Figure 4D). The shift of 1.4 pK_a units is not as pronounced as for a recently analyzed adenine in the catalytic domain 5 of a bacterial self-splicing group II intron (2.4 pK_a units),[28] but still has to be considered relevant for possible acid–base catalysis in the phosphodiester cleavage mechanism of twister. In general,

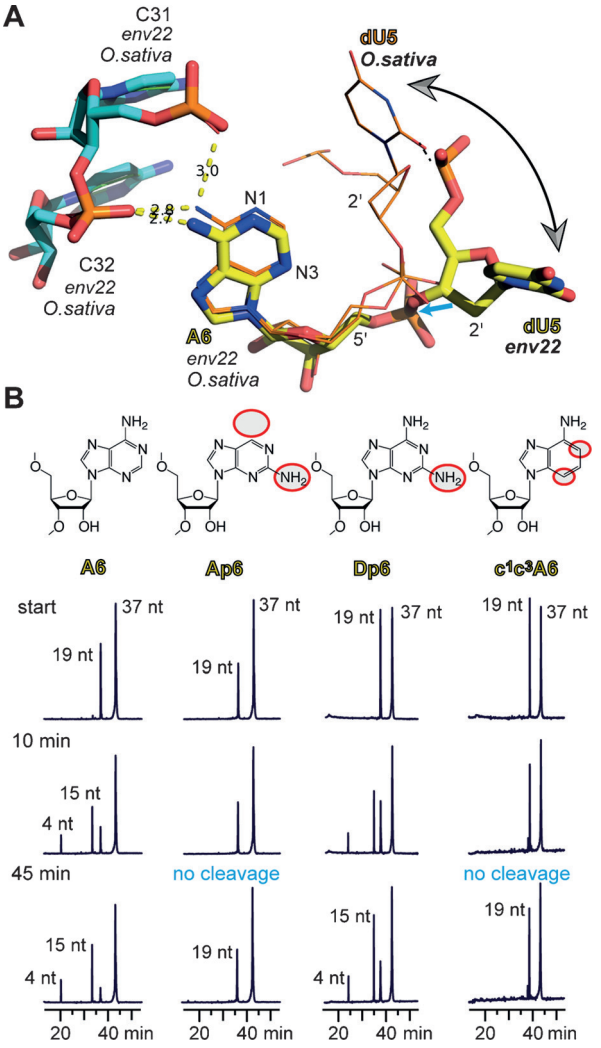

Figure 3. Twister ribozyme activity critically depends on the integrity of the adenine at the scissile phosphate. A) Overlay of X-ray structures PDB 4OJI (*O. sativa,* thin lines) and PDB 4RGF (*env22,* sticks) of the nucleosides at the cleavage step A6-U5 (yellow) highlighting the interaction of A6 with C31–C32 phosphates. The rotation of the off-line oriented U5 (*O. sativa*) to take the in-line position of U5 (*env22*) is indicated by arrow with gray heads. The C2′ of dU5 used for the crystal structure determination is labeled with 2′. Note that the distance between C2′ and N3 is significantly closer (4 Å) for the off-line dU5 than for the in-line orientated dU5 (7 Å). B) The $N6H_2$ group of A6 seems crucial for proper positioning of this nucleoside. Its deletion makes the ribozyme inactive. No cleavage is also observed when the purine nitrogens N1 and N3 are mutated to non-basic carbon atoms (c1c3A). Conditions: 2 mM $MgCl_2$, 100 mM KCl, 30 mM HEPES, pH 7.5, 23 °C.

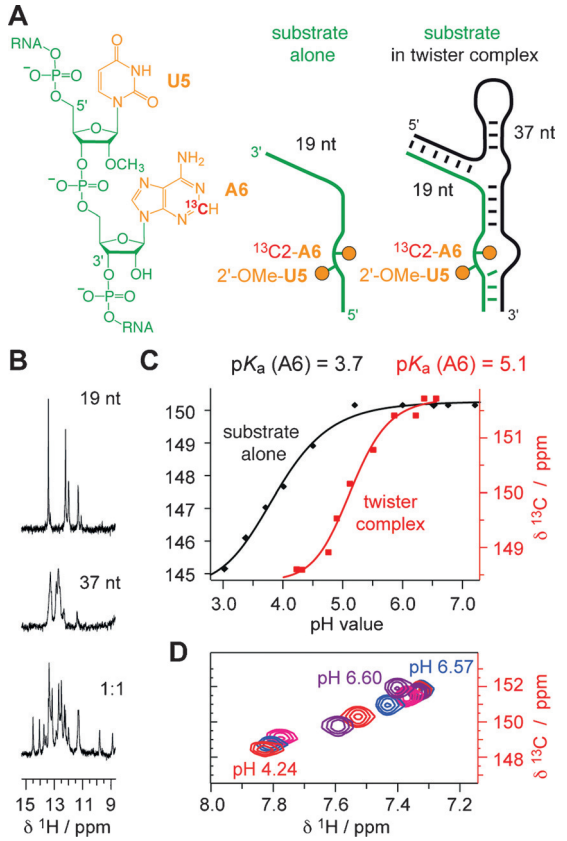

Figure 4. The adenine at the cleavage site of the twister ribozyme has a shifted pK_a. A) Chemical structures of ^{13}C2-labeled A6-2′-OCH_3-U5 (left) and cartoon of the strands used in the NMR experiments. B) Complex formation verified by ^{1}H NMR imino proton spectroscopy. C) Chemical shift changes of ^{13}C2-A6, with changes in the pH value derived from $^1J(^1H,^{13}C)$ HSQC spectra. The lines represent least-squares fits.[33] D) Exemplary $^1J(^1H,^{13}C)$ HSQC spectra of pH-dependent NMR experiments of ^{13}C2-A6 labeled twister. Conditions: *c*(RNA) = 0.5 mM; 100 mM KCl, 2 mM $MgCl_2$, 10 mM Na cacodylate, H_2O/D_2O 9:1, 298 K.

protonation occurs preferentially at N1 of adenine, with minor populations of protonation at N7 and N3.[29] It is one of our future aims to verify the precise location of protonation using the corresponding ^{15}N-single atom labeled A6.[30,31]

In this context, we note that G48 (G62 in *O. sativa* twister ribozyme) has been suggested to act as general base and to activate the attacking 2′-OH.[20] For this reason we intended to determine the pK_a of G48 by using ribozymes with ^{13}C8-labeled G48. Unfortunately, our attempts were unsuccessful. Although we were able to precisely measure the pK_a of guanine in a short RNA (5′-GUUUUA, pK_a of 10.1 ± 0.1) by pH-dependent NMR spectroscopic experiments as described above (Supporting Information, Figure S3), the 37 nt twister RNA degraded rapidly at pH values higher than 9.5 and under the buffer conditions used. Despite several attempts, we were not able to reliably determine the pK_a of G48 in twister by this method. Nevertheless, since no significant pH-dependent chemical-shift change was observed for ^{13}C8-G within the short reference, the 37 nt ribozyme strand and the 37 nt ribozyme strand in complex with a non-cleavable substrate up to a pD of circa 9.0 (Supporting Information, Figure S3), it appears likely that the pK_a of G48 is not shifted towards the neutral pH range.

For the present study, we focused on an additional aspect, namely the influence of the divalent metal ion coordinated to the pro-S_P oxygen of the scissile phosphate as observed in the *env22* but not in the *O. sativa* twister ribozyme structure. We anticipated that cleavage of the S_P diastereoisomer of a phosphorothioate analogue might be accelerated in the

presence of thiophilic metal ions such as Mn^{2+} or Cd^{2+}.[24,30,31] In contrast, we hypothesized that the R_P diastereoisomer might be less efficient in cleavage as the hydrogen bond of G48 to the sulfur atom of the R_P phosphorothioate is expected to be significantly weaker. Consequently, proper positioning of the scissile phosphate towards N1 of G48 might be impaired, and in turn, attaining the active conformation might be hindered.

To test this hypothesis, we synthesized the corresponding phosphorothioate substrate strands, separated the diastereomers by reversed-phase HPLC, assigned R_P and S_P strands according to their order of elution (as reported),[32,33] and conducted the experiments summarized in Figure 5. For these assays, we used the mini-twister ribozyme lacking P1. A single 2-aminopurine nucleoside at the 5′-end was efficiently cleaved from the 15 nt substrate strand with an unmodified phosphate, which served as reference (Figure 5A). We point out that we applied a saturating concentration level of 2 mM Mg^{2+} that was previously determined for this ribozyme.[21] Importantly, substitution of Mg^{2+} by Mn^{2+} ions resulted in comparable cleavage activity (Figure 5A). In contrast, addition of Cd^{2+} ions resulted in significantly reduced cleavage (Figure 5A) albeit the total concentration of divalent metal ions was doubled.

Strikingly, the R_P phosphorothioate diastereomer was not cleaved at all under the 2 mM Mg^{2+} containing standard buffer conditions (Figure 5B, R_P), supporting our hypothesis of an impaired G48 phosphate architecture, most likely due to weaker hydrogen bonding of N1–H···S–P compared to N1–H···O–P. Interestingly, substitution of Mg^{2+} by Mn^{2+} ions resulted in very minor cleavage activity (Figure 5B, R_P). Addition of Cd^{2+} rescued cleavage to some extent (Figure 5B, R_P).

In contrast to the R_P phosphorothioate, the S_P counterpart was cleaved under the 2 mM Mg^{2+} containing standard buffer conditions (Figure 5B, S_P). Cleavage appeared only slightly slower compared to the unmodified phosphate reference strand (Figure 5A). Importantly, substitution of Mg^{2+} by Mn^{2+} ions resulted in significantly increased cleavage activity (Figure 5B, S_P). Likewise, when we added 2 mM Cd^{2+} to the

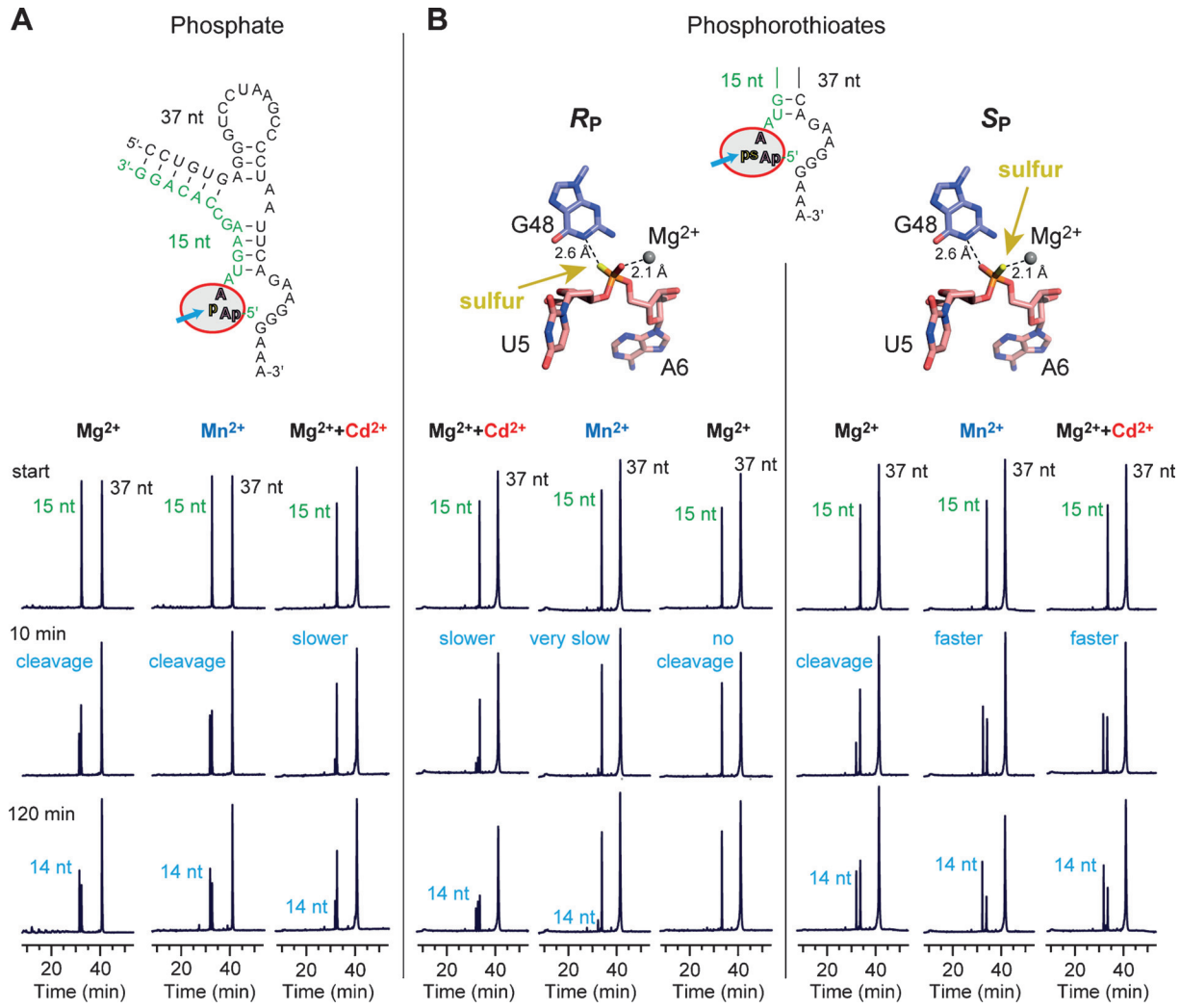

Figure 5. Twister ribozyme activities using phophorothioates as substrates. A) HPLC cleavage assay for the reference phosphate substrate (single 2-aminopurine nucleotide cleavage). B) HPLC cleavage assay for R_P and S_P phosphorothioate substrate. See the text for a detailed interpretation. Conditions: 2.0 mM Mg^{2+} or 2.0 mM Mn^{2+} (plus 2.0 mM Cd^{2+}, if applied), 30 mM HEPES, 100 mM KCl, pH 7.5, 23 °C.

otherwise unaltered standard buffer conditions, we also observed acceleration of the reaction (Figure 5B, S_P). Of note, cleavage yields were clearly increased in the presence of either thiophilic metal ion (Figure 5B, S_P).

Taken together, the observed thioeffect and rescue with thiophilic metal ions support the idea that a divalent metal ion participates in the catalytic mechanism of twister ribozyme cleavage. The findings underline the relevance of the Mg^{2+} ion coordinated to the scissile phosphate that was observed in the in-line aligned *env22* twister structure.[21] We further emphasize the relevance of this metal ion binding site by a new 2.64 Å X-ray structure (PDB code: 5DUN) that was obtained with 2′-OCH_3-U5 at the cleavage site (instead of dU5). This structure (on a *env22* twister ribozyme construct lacking C20 and A56) is of particular interest because it captured a more off-line aligned conformation of the U5-A6 cleavage step (which could reflect the effect of a bulky methyl substitution), but with Mg^{2+} still coordinated to the scissile phosphate (Mg^{2+} to O distances in the 2.1 Å range; Supporting Information, Figure S4, Table S1), a situation that was not seen in previous off-line aligned structures of twister ribozymes.[19,20]

In summary, we have demonstrated three important features relevant to catalysis of the twister ribozyme. First, the phylogenetically conserved stem P1 is dispensable for phosphodiester cleavage. Second, the conserved adenine at the cleavage site possesses a pK_a of 5.1 and hence serves as a candidate to participate in catalysis. The precise role of this adenine, however, remains incompletely understood and further in-depth studies are required to assess its potential for activating the 2′-OH of U5 leading to cleavage of the U5 A6 backbone in the twister ribozyme. Third, we provide further evidence that divalent metal ions are also directly involved in catalysis by showing that Mn^{2+} and Cd^{2+} accelerate S_P phosphorothioate cleavage. Very likely, the twister ribozyme employs a combination of two general strategies of RNA catalysis for phosphodiester cleavage, namely metal ion catalysis and nucleobase-assisted acid–base catalysis.[34–46] Further dedicated investigations and well-designed experiments will be tailored to fully explore and understand the mechanistic intricacies of this fast-cleaving ribozyme class.[49]

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