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Cellular Small Molecules Contribute to Twister Ribozyme Catalysis

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Abstract

The number of self-cleaving small ribozymes has increased sharply in recent years. Advances have been made in describing these ribozymes in terms of four catalytic strategies: α describes in-line attack, β describes neutralization of the non-bridging oxygens, γ describes activation of the nucleophile, and δ describes stabilization of the leaving group. Current literature presents the rapid self-cleavage of the twister ribozyme in terms of all four of these classic catalytic strategies. Herein, we describe the non-specific contribution of small molecules to ribozyme catalysis. At biological pH, the rate of the wild-type twister ribozyme is enhanced up to 5-fold in the presence of moderate buffer concentrations, similar to the 3 to 5-fold effects reported previously for buffer catalysis for protein enzymes. We observe this catalytic enhancement not only with standard laboratory buffers, but also with diverse biological small molecules, including imidazole, amino acids, and amino sugars. Brønsted plots suggest that small molecules assist in proton transfer, most likely with δ catalysis. Cellular small molecules provide a simple way to overcome the limited, functional diversity of RNA and have the potential to participate in the catalytic mechanisms of many ribozymes *in vivo*.

Graphical Abstract

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ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website.

Notes

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INTRODUCTION

Small nucleolytic ribozymes (RNA enzymes) promote self-cleavage of the phosphodiester linkage between adjacent nucleotides.^{1,2} This reaction is accomplished by activation of the 2'-OH nucleophile, which attacks the neighboring scissile phosphate to give products with 2',3' cyclic phosphate and 5'-OH termini. To catalyze this reaction, ribozymes utilize four distinct catalytic strategies: in-line arrangement of the 2'O-P-5'O atoms (α catalysis), neutralization of charge buildup on the non-bridging oxygens of the scissile phosphate (β catalysis), activation of the 2'-OH nucleophile (γ catalysis), and stabilization of the 5'O leaving group (δ catalysis).^{1,3,4} While some ribozymes may use all of these strategies to accomplish self-cleavage, many appear to use only a subset.^{3,4} Ribozymes have developed unique molecular means to contribute to these four strategies. For instance, γ catalysis can be enhanced by acidifying the 2'-OH nucleophile and/or releasing the 2'-OH from inhibitory interactions.⁴⁻⁶

An additional mode of catalysis has been reported for several protein enzymes, including carbonic anhydrase and CFA synthase, in which buffer catalyzes the reaction; in carbonic anhydrase a variety of buffers are operative, while in CFA synthase the buffer must be bicarbonate.⁷⁻¹⁰ For carbonic anhydrase, buffer participates in a proton shuttle, as supported by mechanistic, structural, and theoretical studies.^{9,11} For CFA synthase, no explicit role has been assigned to buffer, but it is suspected to act as a general base, or provide electrostatic stabilization to a carbocation intermediate.¹⁰ In these protein enzymes, buffer contributes ~3 to 5-fold to catalysis. An open question is whether RNA enzymes use buffers in their mechanisms.

The twister ribozyme is recently discovered and self-cleaves between U-1 and A1.¹² Since its discovery, intensive studies have been carried out, which have led to crystal structures,¹³⁻¹⁶ mechanistic investigations,^{12,13,15-17} and computational analyses.^{18,19} This flurry of activity has led to important advances, but disagreements remain about key aspects of the mechanism.² Crystal structures reveal that G53 is near the 2'-OH nucleophile, while the *syn* A1 is in the vicinity of the 5'O leaving group (Table S1).¹³⁻¹⁶ It is generally agreed upon that the twister ribozyme utilizes α catalysis, β catalysis via the N2 of G53, and γ catalysis via the N1 of the same guanine.^{1,4,17} The nature of δ , or general acid, catalysis is less certain. Mechanistic and mutagenic studies utilizing 3-deaza-adenine suggest that the N3 of A1 may act as the general acid in the reaction,¹⁷ which is appropriately positioned in a crystal structure.¹⁴ However, protonated A1(N3) has an unperturbed pK_a of just 1.5,²⁰

similar to the phosphodiester backbone; and while NMR measurements on an *env22* twister report an upwardly perturbed pK_a , its experimental value is still just 5.1.¹⁶ It is thus unclear whether A1(N3) can serve as a general acid at physiological pH. Moreover, in the available crystal structures, the A1 nucleotide is positioned from 3.2 to 5.3 Å from the 5' O leaving group, with longer distances coinciding with crystallographic pHs of 7.9 to 8.0 (Table S2).^{13–16} The multichannel mechanism proposed herein reconciles these observations.

RESULTS AND DISCUSSIONS

We began our study by measuring rate-pH profiles for the *env9* twister ribozyme, secondary structure provided in Figure S1.¹² We initially describe data with a Tris-acetic acid-MES ternary buffer system (TAM), which has constant ionic strength at all pH.²¹ At biological concentrations of Mg^{2+} of 0.5 and 1.0 mM,²² the low pH arms of the rate-pH profiles are log-linear, with slopes of ~ 1 (Figure 1, Table S3). The 0.5 mM Mg^{2+} rate-pH profile showed simple plateau behavior up to pH 8.5 and was therefore fit to a standard binding isotherm with a single ionization, which returned a pK_a of 6.8 ± 0.1 (Figure 1A, inset). The shape and pK_a of this rate-pH profile, including a decrease at high pH, are similar to that reported by Lilley and coworkers on the *env9*-related ES2 twister construct.¹³

Because the microscopic pK_a for protonated A1(N3) was directly measured to be just 5.1,¹⁶ we hypothesized that buffer might contribute to the reaction at biological pH where the N3 would be mostly deprotonated. To test this idea, we repeated the rate-pH profiles in the presence of four individual buffers. At 0.5 and 1.0 mM Mg^{2+} , over the low pH range of 5.5 to 6.5, the individual buffers and the TAM buffer system were superimposable within experimental error (Figure 1A, B). However, at higher pH and/or in biological concentrations of Mg^{2+} ions, the two sets of buffers showed divergent behavior. At 0.5 mM Mg^{2+} , the reactions in individual buffers and TAM buffers displayed bell-shaped behavior between pH 6.5 and 9.0, with a more distinct bell shape for the reactions in individual buffers. At 1 mM Mg^{2+} , reactions in individual buffers and TAM buffers showed similar shapes, but the individual buffers reacted faster than the TAM buffers at pH 7 and higher. Thus, over the pH range of 6.5 to 9.0, which includes biological pH, the reaction depends on buffer identity. Shapes of these rate-pH profiles can be understood as arising from buffer-catalyzed channels becoming operative at neutral pH (see below).

Given that rate-pH profiles of ribozymes often depend on Mg^{2+} ,^{23–26} we next measured the dependence of the rate on Mg^{2+} concentration at pH 5.5, 6.5, and 7.5. The data at the higher pH showed apparent K_D s of 1.9 ± 0.1 mM, and 2.6 ± 0.2 mM and Hill coefficients of 1.8 ± 0.1 and 1.7 ± 0.1 at pH 6.5 and 7.5, respectively (Figure S2, Table S4). We then measured rate-pH profiles in 10 mM Mg^{2+} , which is saturating according to these K_D values and Hill coefficients. Strikingly, the rate was now dependent on buffer identity throughout the entire pH range, even in the low pH arm, with reaction in TAM buffers faster than in individual buffers (Figure 1C). We also performed kinetic solvent isotope experiments (KSIE), which returned values of 4.1 ± 0.9 and 5.0 ± 1.2 at 1 and 10 mM Mg^{2+} , respectively, indicating that proton transfer is rate-limiting under both conditions (Table S5). The strong buffer dependence throughout the entire pH range motivated us to investigate the dependence of the reaction on buffer concentration and identity.

We used 30 mM of each individual buffer; and 100 mM Tris, 50 mM acetic acid, and 50 mM MES in the TAM buffer system. We hypothesized that the higher buffer concentrations in the TAM buffers may have been responsible for their faster rate in 10 mM Mg^{2+} . Thus, we investigated the dependence of the reaction on buffer concentration, varying Tris buffer from 0 to 170 mM in a background of 30 mM HEPES. Between pH 6.5 and 7.5 at 10 mM Mg^{2+} , Tris enhanced the rate 2.2 ± 0.3 to 2.6 ± 0.4 -fold (Figure 2). These rate increases are similar to previous ones of 3–5 fold, observed in carbonic anhydrase and CFA synthase.^{9,10} Additionally, k_{obs} values at ~100 mM Tris and pH 6.5, 7.0, and 7.5 match closely with k_{obs} values in TAM buffer, supporting Tris as the major contributor in TAM buffer. Moreover, at pH 6.5, with 10 mM Mg^{2+} in 10 and 100 mM Tris buffer, we found KSIEs of 5.0 ± 1.2 and 4.4 ± 1.0 respectively (Table S5), supporting Tris buffer catalyzing self-cleavage through proton transfer.

Next, we tested whether biologically relevant small molecules can catalyze twister ribozyme self-cleavage. We chose to investigate amino acids and amino sugars, as they are prevalent in most organisms, with concentrations up to several hundred millimolar.²⁷ Controls showed that ionic strength is not responsible for effects of amino acids on rate (Table S6). Several metabolites have a marked impact on the rate of self-cleavage, notably imidazole and histidine, which have $\text{p}K_{\text{a}}$ values near 7, increase the rate from 2.2 to 4.0-fold at pH 6.5 and 7.5 (Figure 3). The primary amine-containing biological small molecules glycine, lysine, and glucosamine, which have $\text{p}K_{\text{a}}$ values around 9, also stimulated the reaction, with rate enhancement at pH 7.5 ranging from 1.2 to 2.1-fold, similar to the effects from Tris buffer. The only small biological molecule tested that did not enhance the rate at pH 7.5 was arginine. On the one hand, arginine's high $\text{p}K_{\text{a}}$ of 12.5 is not tuned to proton transfer, but on the other it also has a primary amine of $\text{p}K_{\text{a}}$ similar to other amino acids and so might be expected to stimulate the reaction. The absence of stimulation with arginine is unclear but could be due to compensating effects since arginine often interferes with RNA function.²⁸ Overall, these data suggest that a number of small molecules in the cell could assist ribozymes in catalysis.

Effects of small molecules on cleavage suggested that the rate of the reaction might depend on the $\text{p}K_{\text{a}}$ of the small molecule. We therefore measured dependence of rate on $\text{p}K_{\text{a}}$ for seven small molecules varying in $\text{p}K_{\text{a}}$ from 6.0 to 9.6 at either pH 6.5 or 7.5. Figure 4 provides a Brønsted plot of $\log k_{\text{obs}}$ versus the $\text{p}K_{\text{a}}$ of the small molecule at pH 7.5 and 10 mM Mg^{2+} , with k_{obs} extrapolated to 100% small molecule protonation (panel A) or deprotonation (panel B). For protonation, we observe linear behavior, with a slope of -0.46 ± 0.09 (R^2 of 0.88), supporting a model in which small molecules contribute to catalysis under these conditions (Figure 4A). The analogous plot of $\log k_{\text{obs}}$ versus $\text{p}K_{\text{a}}$ of the small molecule extrapolated to 100% small molecule deprotonation also shows linear dependence, which is expected owing to the principle of kinetic ambiguity,^{29–31} with a slope of 0.54 ± 0.09 (R^2 of 0.85) (Figure 4B). Brønsted plots at pH 6.5 are also linear, but with the slope for protonation is reduced to -0.23 ± 0.07 (Figure S3), which is consistent with the above notion that small molecule-mediated protonation plays a lesser role at low pH. Finally, we note that 2-methylimidazole stimulates the reaction maximally, at 4.7-fold (Figure 3), which is nearly the same as the 5.0-fold stimulation by imidazole in the mechanism for the protein enzyme carbonic anhydrase.⁹

The data presented above support a multichannel mechanism for the twister ribozyme (Figure 5A). Overlapping of the rate-pH profiles for the different buffer systems at low pH and low Mg^{2+} concentration suggests that under these conditions buffer is not involved in the reaction and A1(N3H⁺) can directly protonate the 5' O leaving group, as proposed by Lilley, York, and co-workers, as well as found in our low pH molecular dynamics simulations in which we directly protonated A1(N3).^{14,17–19} We refer to this as ‘channel 1’, k_1 (Figure 5B). This channel is also supported by the absolute dependence of the rate on the presence of N3 at A1.¹⁷ At pH above 6.5, non-overlapping of the rate-pH profiles for different buffer systems (Figure 1) suggests that buffer participates in the reaction under these conditions.¹⁵ We refer to these as ‘channel 2’ and ‘channel 3’ (Figure 5), where channel 3 differs from channel 2 in that two Mg^{2+} ions are bound, as supported by the Hill coefficient in our Mg^{2+} titrations at higher pH (Table S4) and by higher pH crystal structures (Table S2). For instance, twister ribozyme crystal structures by Patel and co-workers, which were solved at pH 7.9, uncovered two ions: one Mg^{2+} ion, 7.8 Å from the A1(N3), which may electrostatically disfavor proton binding here, and a second unassigned ion, 10.4 Å away.¹⁵ Influence of Mg^{2+} on the reaction is further supported by dependence of the rate constant on buffer identity at all pH values in the presence 10 mM Mg^{2+} (data in Figure 1C; model in Figure 5A, 5C). Near neutral pH, the influence of buffer is between 2 to 5-fold as compared to the direct protonation-catalyzed channel (Figure 3), suggesting that 50–80% of the reaction flux goes through the buffer-catalyzed channel under biological conditions (see Supporting Information).

A key question is whether the buffer assists in γ catalysis, involving the 2'-OH, or in δ catalysis, involving the 5' O. Inspection of available twister ribozyme crystal structures reveals that the A1(N3) to 5' O leaving group distance increases somewhat with pH (Figure S4, Table S2). For example, the closest distance of 3.2 Å is found at pH 4.6, and the longest distance of 5.3 Å is found at pH 7.9.^{14,15} Moreover, molecular dynamic studies reveal that a single water molecule resides between A1(N3) and the 5' O a significant fraction of the time when A1(N3) is deprotonated.¹⁹ On the other hand, the distance from G53(N1) to U-1(2'C) does not trend with pH across these six structures (Table S2) suggesting that γ catalysis is not buffer-mediated. Thus, the most likely scenario is that at biological pH, buffer assists in δ catalysis, which may be necessary owing to extensive deprotonation of the A1(N3H⁺) at this pH. However, we cannot rule out other scenarios such as the small molecules influencing the pK_a of A1.

We investigated whether pyrazole, which has a very low pK_a of 2.5, could stimulate the reaction. Under all conditions tested, including pH 6.5 and 7.5 at 10 mM Mg^{2+} , pyrazole had no effect on rate (Figure S5). Because pyrazole is essentially deprotonated at these pH values, this suggests that biological small molecules assist the reaction in their protonated form, i.e. by assisting δ catalysis.

One possible model for buffer catalysis is provided in Figure 5C, in which buffer assists protonation of the leaving group through a proton shuttle from the water bridged between A1(N3) and the 5' O. This relay could involve additional waters. Studies from Lilley and coworkers show that the A1(N3) is critical even at higher pH,¹⁷ consistent with such a water

coordination. Indeed, water and water networks have been proposed to play roles in other ribozymes.^{32,33}

Previous studies on other ribozymes, such as the hairpin, HDV, and hammerhead, showed small molecule rescue, but these were performed in the background of either an abasic change, a nucleotide deletion, or mutation of a critical residue.^{34–37} Addition of small molecules such as imidazole and nucleobases, in the wild type background of these particular ribozymes has no effect.^{34,35,37} In contrast, our studies herein on the twister ribozyme show up to 5-fold rate enhancement in the pure wild-type background. The findings presented are, to our knowledge, unique in the ribozyme literature. This effect may be possible because the twister ribozyme has a relatively solvent-exposed active site (Figure S6).

CONCLUSIONS

The twister ribozyme is able to react by three channels: one involving direct proton transfer from A1(N3H⁺) to the leaving group, populated at lower pH, and two channels involving buffer-mediated proton transfer from a water at higher pH and/or Mg²⁺ concentration, comparable to those found in a cell. Under biological conditions, buffer contributes at least as much as direct protonation to rate enhancement. Our data suggest that the small molecule can be any of the ubiquitous amine- or imine-containing metabolites in the cell. Recruitment of small molecules from the cellular milieu is similar to that found in the classic mechanism in the protein enzyme carbonic anhydrase, both in terms of magnitude and proton shuttle mechanism. Buffer catalysis in RNA enzymes reflects both the opportunistic nature of evolution and the versatility of RNA to overcome its intrinsically limited functional diversity. Proton shuttles may play roles in other RNA enzymes, including large RNA enzymes such as the group I and group II introns, RNase P, as well as the ribosome and spliceosome, where general acids and bases for direct proton transfer are not obvious from inspection of crystal structures.

Supplementary Material

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Figure 1.

Dependence of twister self-cleavage on buffer. Plots of $\log(k_{\text{obs}})$ versus pH for self-cleavage of the *env9* twister ribozyme at either (A) 0.5 mM, (B) 1 mM or (C) 10 mM MgCl_2 with 30 mM individual buffers (open circles) or 100 mM Tris, 50 mM acetic acid and 50 mM MES (TAM buffer, closed circles). The Figure 1A inset is the TAM buffers, up to pH 8.5, fit to a standard model with a single ionization giving a $\text{p}K_a$ of 6.8 ± 0.1 .

Figure 2.

Dependence of the twister ribozyme on Tris concentration. Conditions are 10 mM MgCl_2 at either (A) pH 6.5, (B) pH 7.0 and (C) pH 7.5. Reactions are fit to the Hill equation with a fixed Hill coefficient of 1. Buffer systems consisted of a combination of 30 mM HEPES and variable Tris concentration, 10 to 170 mM Tris.

Figure 3.

Cleavage of the twister ribozyme in various small biological molecules. Non-normalized k_{obs} values of the twister self-cleavage reaction in the presence of 10 mM Mg^{2+} . Reactions were conducted at either pH 6.5 (black) or pH 7.5 (grey). The “HEPES” trials contain only 5 mM HEPES with no additional small molecules while the remainder contain 5 mM HEPES and 120 mM of the specified small molecule except Tris experiments contained 30 mM HEPES and 100 (pH 6.5) or 120 mM (pH 7.5) Tris.

Figure 4.

Brønsted plots of seven small biological molecules. Conditions are pH 7.5 and 10 mM Mg^{2+} where the k_{obs} values were extrapolated to either (A) 100% protonated, or (B) 100% unprotonated. The graphs show linear behavior with slopes of -0.46 ± 0.09 and $+0.54 \pm 0.09$ for panels (A) and (B) respectively. Identities of the small molecules are provided in Figure S3.

Figure 5.

Proposed multichannel mechanism of the twister ribozyme. (A) Buffer-free and buffer-promoted channels, where high Mg^{2+} conditions also promote the small molecule-catalyzed mechanism. “A⁺” is A1 protonated at the N3 and “Buf⁺” depicts any small molecule of appropriate $\text{p}K_{\text{a}}$. (B) Non-small molecule-catalyzed channel (Channel 1) at low concentration of Mg^{2+} . (C) Proposed small molecule-catalyzed channels (Channels 2 and 3).