The Hammerhead Ribozyme Catalyzes the Deglycosylation of 2′-Mercaptocytidine

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Contemporary enzymes sometimes exhibit catalytic promiscuity, whereby an enzyme that evolved to catalyze one chemical transformation can also catalyze an alternative reaction at a low level.[1] For example, chymotrypsin normally catalyzes an amidase reaction but also possesses a low level of phosphonodiesterase activity at the active site.[2] Such alternative activities may have played an important role in evolution by providing a mechanism by which new enzyme functions could arise.[1] Thus, uncovering and understanding such activities might reveal clues about the pathway of evolution and the potential of past and present enzymes to serve as precursors to new enzymes and may help guide engineering of enzymes with new activities.[1] Here we report an example of an RNA enzyme that exhibits catalytic promiscuity.

The hammerhead ribozyme (HH) is a small, self-cleaving RNA that catalyzes a phosphotransesterification reaction in which a distinct 2′-OH group attacks the adjacent phosphate to produce cleavage products containing 2′,3′-cyclic phosphate and 5′-OH termini.[3] As an initial study to evaluate the potential of 2′-mercaptonucleosides as biochemical tools, we probed the active site of the HH by replacing the 2′-OH nucleophile with a 2′-mercapto (−SH) group. Rather than facilitating attack of the 2′-SH group on the adjacent 3′-phosphodiester, analogous to the normal biological reaction, the ribozyme catalyzes a very different reaction in which the 2′-SH group attacks the adjacent 1′-C to displace the heterocycle.

We synthesized two substrates ($^{5'}$dGGGAACGTC$_{2'X}$GTC-GTCGCC$^{3'}$) for the HH16 ribozyme containing 2′-deoxynucleotides at all positions except for a ribocytidine (X = OH; dHH16OH) or a 2′-mercaptocytidine (X = SH; dHH16SH) at the cleavage site.[4,5] We chose the HH16 ribozyme because it is well-characterized,[6] and we used substrates in the 2′-deoxyribose background to simplify analysis of the products.

The substrates were radiolabeled at their 3′-ends using [α-^{32}P]-cordycepin (Co) and terminal deoxynucleotidyl transferase. We carried out HH reactions in 50 mM NaMOPS (pH 7.5) at 25 °C. In the presence of saturating ribozyme and 10 mM Mg^{2+}, dHH16OH cleaved as expected to give a single radiolabeled product **P2** (Figure 1A, lane 7) that comigrated with an independently synthesized standard, $^{5'}$dGTCGTCGCC*pCo$^{3'}$ (standard A; lane 8; *p indicates a ^{32}P-phosphate). No product formed in control reactions lacking divalent metal ions or ribozyme or in reactions with an inactive mutant HH, in which the essential A14 was modified to G (A14G; Figure 1A, lanes 2−6).[8] In control

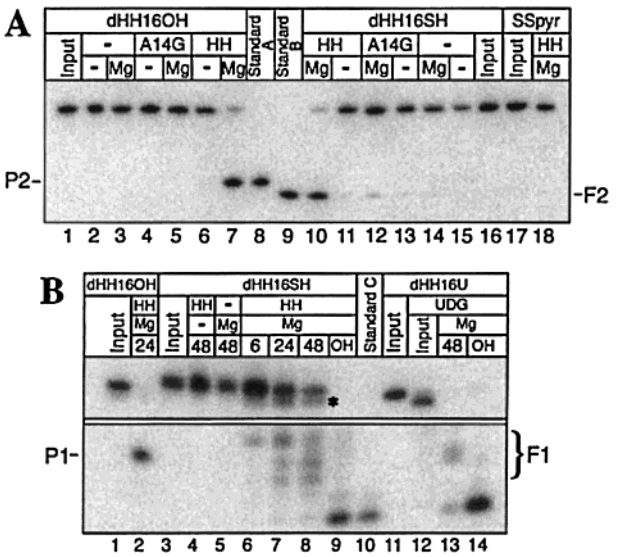

Figure 1. Hammerhead reactions with 3′- and 5′-radiolabeled substrates (A and B, respectively). All reactions contained 50 mM NaMOPS (pH 7.5) and 1 mM TCEP and were incubated at 25 °C. (A) dHH16OH, dHH16SH and dHH16SSpyr, in which the 2′-S is protected as a 2-pyridyl disulfide (SSpyr), were incubated for 36 h with no HH, an inactive HH (A14G) or wild-type HH in the presence or absence of 10 mM Mg^{2+} as indicated. (B) dHH16OH (lane 1) and dHH16SH (lane 3) were incubated in the presence or absence of HH and Mg^{2+} for 6, 24, or 48 h as indicated. DHH16U (lane 10) was treated with UDG at 37 °C for 20 min (lanes 12−14) before incubation with 10 mM Mg^{2+} for 48 h (lanes 13−14). The reactions in lanes 8 and 13 were treated with 0.1 M NaOH at 90 °C for 10 min (lanes 9 and 14, respectively). The middle section of the gel was removed to conserve space.

reactions with dHH16SH, no **P2** formed (Figure 1A, lanes 11−15), but a faster migrating product **F2** was weakly visible that gave the identical S1 nuclease digestion pattern (see Supporting Information) as the independently synthesized standard, $^{5'}$p-dGTCGTCGCC*pCo$^{3'}$ (standard B; lane 9). The appearance of **F2** is consistent with the slow fragmentation observed previously for an oligonucleotide containing a 2′-mercaptocytidine residue.[4] In the presence of saturating ribozyme and 10 mM Mg^{2+}, dHH16SH resisted phosphotransesterification but gave rise to **F2** at a significantly accelerated rate (Figure 1A, lane 10) compared to the control reactions.[8] The ribozyme was unable to catalyze the formation of the phosphotransesterification product **P2** under any of the conditions tested, including a range of pHs, temperatures, and Mg^{2+} concentrations. This lack of **P2** formation was not unexpected because a 2′-SH is a much weaker nucleophile than a 2′-OH group toward the adjacent 3′-phosphate.[9]

Under basic conditions 2′-mercaptouridylyl-(3′-5′)-uridine and 2′-deoxy-2′-mercaptouridine 3′-(*p*-nitrophenyl phosphate) undergo fragmentation to give uridine 5′-phosphate and *p*-nitrophenyl phosphate, respectively, and uracil.[9,10,11] The release of uracil implies a mechanism involving attack of the 2′-S at the 1′-C to displace the heterocycle. The chemical pathway for fragmentation of dHH16SH may involve analogous glycosidic bond cleavage to release cytosine, followed by backbone cleavage of the resulting abasic 1, 2-episulfide to generate the 5′-phosphorylated product,

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(5) To maintain the integrity of the mercapto group was not compromised during radiolabeling and purification, we protected it as a 2-pyridyl disulfide (dHH16SSpyr) before these steps. We generated dHH16SH in situ as described in ref 4.

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(8) We also performed the entire set of experiments for the HH8 system (see Supporting Information).

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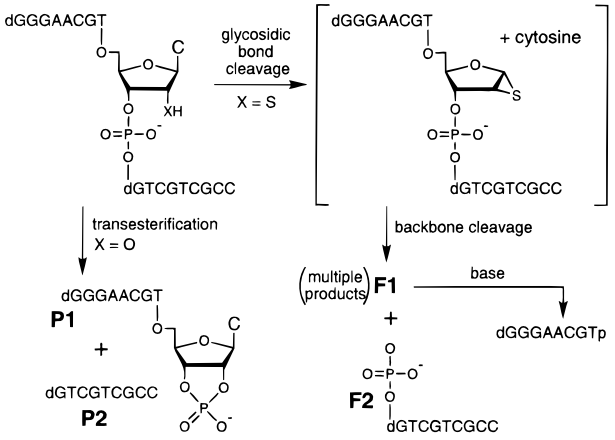
Scheme 1



F2 (Scheme 1).[4,9,10] We confirmed the formation of free cytosine by monitoring the reaction using HPLC (see Supporting Information). Consistent with a mechanism that involves nucleophilic attack by the 2′-S, dHH16SSpyr, in which the 2′-S is blocked as a disulfide, showed no tendency to fragment (Figure 1A, lane 18) in the presence of HH and Mg^{2+}.

To explore the mechanism of fragmentation further, we carried out analogous experiments with 5′-radiolabeled substrates (Figure 1B). Similar to the reactions with 3′-radiolabeled substrates, no products resulted during incubation of either substrate without ribozyme or Mg^{2+} (data not shown and Figure 1B, lanes 4 and 5). In the presence of HH and Mg^{2+}, dHH16OH formed only one product (Figure 1B, lane 2), presumably **P1**, which terminates with a 2′,3′-cyclic phosphate (Scheme 1). The 5′-product **F1** from dHH16SH usually migrated as a smear in the electrophoresis gel. However, electrophoresis through a longer gel resolved the smear into multiple species of unknown identity (Figure 1B, lane 8; Scheme 1), none of which comigrated with **P1**. Additionally, these electrophoresis conditions revealed a species that migrated slightly faster than dHH16SH (dHH16S*; indicated by the asterisk in Figure 1B, lanes 6−8).[12] The possibility that this new species could be an abasic intermediate formed by deglycosylation of 2′-mercaptocytidine led us to generate for comparison a substrate analogue (dHH16Ab) that contained an abasic 2′-deoxyribose moiety at the cleavage site. We generated dHH16Ab by synthesizing an oligonucleotide for HH16 that contained a 2′-deoxyuridine at the cleavage site, $^{5'}$dGGGAACGTUGTCGTCGCC$^{3'}$ (dHH16U), followed by incubation with uracil DNA glycosylase (UDG) to remove uracil from the 2′-deoxyuridine residue. dHH16S* and dHH16Ab are strikingly similar in three ways: (1) they migrate slightly faster than the precursors from which they were derived (Figure 1B, lanes 8, 11, and 12), (2) under the HH reaction conditions, they undergo cleavage of the ribose phosphate backbone at similar rates to give the 3′-product **F2** (data not shown) and multiple 5′-products (Figure 1B, lanes 8 and 13), and (3) base treatment (0.1 N NaOH, 90 °C, 5 min) converts the 5′-products derived from dHH16Ab (Figure 1B, lane 14) and dHH16S* (lane 9) primarily to $^{5'}$*pdGGGAACGTp$^{3'}$ (Standard C; lane 10), suggesting by analogy that the 5′-products from dHH16S* contain a 3′-sugar moiety that is susceptible to further elimination under basic conditions (Scheme 1).[13] These results, coupled with the observation that cytosine is released at the same rate that dHH16S* is formed (see Supporting Information), strongly suggest that dHH16S* is an abasic intermediate in the fragmentation reaction.[14]

In the presence of 10 mM Mg^{2+} and HH, the intermediate dHH16S* forms at a rate of 1.2×10^{-3} min^{-1} and undergoes backbone cleavage at a rate of 4×10^{-4} min^{-1} (unpublished results). In the absence of HH, dHH16SH fragments much more slowly (2×10^{-5} min^{-1}) and dHH16S* cannot be observed, suggesting that the deglycosylation step is rate-limiting in the background reaction. We infer that the HH facilitates the fragmentation of dHH16SH by catalyzing the deglycosylation step ~60-fold. It appears unlikely that the HH also catalyzes backbone cleavage of dHH16S* because backbone cleavage of dHH16Ab occurs at the same rate (4×10^{-4} min^{-1}) as dHH16S* and is not catalyzed by the ribozyme (data not shown).

HH catalysis of the fragmentation reaction requires some of the same structural features as the native phosphotransesterification reaction. Substitution of the cytidine at the cleavage site with uridine reduces the phosphotransesterification rate by 25-fold,[7] while substitution of 2′-mercaptocytidine with 2′-mercaptouridine reduces the fragmentation rate to that of background (data not shown). Substitution of A14 of the ribozyme to G abolishes both activities, as does the absence of metal ions. However, unlike phosphotransesterification, fragmentation still occurs if spermine or cobalt hexamine, cations that are known to stabilize RNA structure, replace the divalent metal ions (see Supporting Information). Perhaps the HH facilitates fragmentation by binding dHH16SH in a conformation that favors reaction and/or by fortuitous use of active site features.

In conclusion, the HH facilitates fragmentation of an oligonucleotide containing 2′-mercaptocytidine by catalyzing nucleophilic attack of the 2′-sulfur at the 1′-carbon to displace cytosine. Although catalysis of this "deglycosylation" reaction is modest (~10^2-fold), the reaction is distinct from the normal HH phosphotransesterification reaction and provides an example of catalytic promiscuity at an RNA active site. These results further expand the catalytic repertoire of RNA[15] and have significant implications for the RNA world.[16] During this hypothetical era of evolution, metabolically complex organisms supposedly used RNA as the cell's primary genetic and catalytic machinery. Our findings provide a chemical basis for two possible mechanisms by which ribozymes of that era might have achieved catalytic complexity: (1) catalytic promiscuity could have played an important role in the creation of new ribozyme activities, and (2) RNA could have used sulfur nucleophiles to catalyze reactions analogous to those of contemporary enzymes that use sulfur nucleophiles, including cysteine proteases,[17] tyrosine phosphatases,[18] thymidylate synthetases,[19] etc.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) This was also true for reactions with 3′-radiolabeled dHH16SH (see Figure C of Supporting Information).

(13) Abasic sites in DNA undergo cleavage of the phosphodiester backbone through a β-elimination mechanism. β-elimination occurs because the 2′-H's at abasic sites are acidic in the ring-opened aldehyde form of the sugar. Removal of the 2′-H generates a resonance stabilized enolate anion that can eliminate the 3′-phosphoryl group. Following this elimination, the 4′-H of the newly formed β-enal becomes acidic and may be removed to create a new enolate that can eliminate the 5′-phosphoryl group. See: Mazumder, A.; Gerlt, J. A. Abalson, M. J.; Stubbe, J.; Cunningham, R. P.; Withka, J.; Bolton, P. H. *Biochemistry* **1991**, *30*, 1119.

(14) The mechanism for backbone cleavage of dHH16S* might be similar to the β-elimination mechanism that gives rise to backbone cleavage at abasic sites in DNA.[13] In the case of a 1,2-episulfide (Scheme 1), the thioacetal linkage at C-1′ could hydrolyze to the abasic 2′-mercaptoribose, which, like an abasic site, would be poised to rearrange to the ring-opened aldehyde and undergo β-elimination. An alternative mechanism to displace the 3′-phosphoryl group could occur if the aldehyde rotates about the C2′−C3′ bond and the 2′-mercapto group attacks at C3′ in an S_N2-like reaction. Under basic conditions, *O*-phosphorylated derivatives of 2′-mercaptoethanol react in this way. See: Gay, D. C.; Hamer, N. K. *J. Chem. Soc. B* **1970**, 1123.

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