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Active Site Labeling of G8 in the Hairpin Ribozyme: Implications for Structure and Mechanism

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Abstract: There is mounting evidence that suggests that general acid/base catalysis is operative in the hairpin ribozyme, with analogy to the protein enzyme RNaseA. Nevertheless, the extent of general base catalysis as well as the identity of the specific chemical groups responsible remains the subject of some controversy. An affinity label has previously been used to alkylate histidine 12 (His12), the active general base in RNaseA. To date, no such experiment has been applied to a ribozyme. We have synthesized the analogous affinity label for the hairpin ribozyme with an electrophilic 2′-bromoacetamide group in lieu of the 2′-hydroxyl (2′OH) at the substrate cleavage site and show that guanosine 8 (G8) of the hairpin ribozyme is specifically alkylated, most likely at the N1 position. This evidence strongly implicates N1 of G8 in active site chemistry. By direct analogy to RNase A, these findings could be consistent with the hypothesis that deprotonated G8 residue functions as a general base in the hairpin ribozyme. Other mechanistic possibilities for N1 of G8 such as indirect general base catalysis mediated by a water molecule or transition state stabilization could also be consistent with our findings.

Introduction

Small nucleolytic ribozymes such as the hairpin ribozyme catalyze sequence specific RNA cleavage and, in some cases, the reverse reaction, RNA ligation.[1] The cleavage reaction catalyzed by the hairpin ribozyme proceeds through "in-line" nucleophilic attack by a 2′OH on the adjacent scissile phosphate, expelling the 5′OH of the 3′-product and generating a 5′-product terminating in a 2′−3′ cyclic phosphodiester.[2,3] Despite a wealth of biochemical data and, more recently, high-resolution X-ray structural data,[4] the detailed catalytic mechanism of the hairpin ribozyme, in particular with regards to the chemical functionality responsible for catalysis, remains the subject of considerable debate.[5−8]

The protein RNaseA, which catalyzes RNA cleavage by the same phosphoryl transfer reaction to the 2′OH, has been studied in detail for decades. RNaseA is unquestionably M^{2+}-independent and makes use of the imidazole side chains of two active site histidine residues for general acid/base catalysis (Figure 1).[9] Imidazole, with a p$K_a \approx 7$, is particularly well suited to the role of general acid/base catalysis at physiological pH.[10] Conversely, free ribonucleotides contain no functional groups

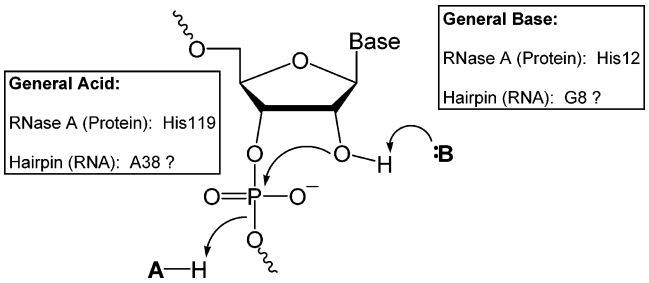

Figure 1. A simplified general acid/base-catalyzed mechanism for RNA cleavage. The residues responsible for general acid/base catalysis in RNaseA are shown along with their putative nucleobase counterparts in the hairpin ribozyme.

with pK_a's between 5 and 9,[11] and as such, the nucleobases would appear ill suited for such catalysis. This fact is thought to be at least partially responsible for the general catalytic inferiority of ribozymes compared to the much more functionally diverse protein enzymes.[12,13] The local environment of a folded RNA structure can perturb nucleobase pK_a's toward a value of 7.4 such that competent general acid/base catalysis by ribozymes may be conceivable.[14−19] Other studies such as the rescue of

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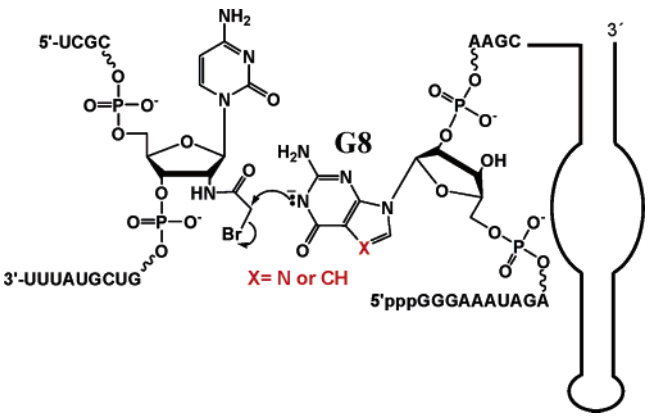
abasic ribozymes with exogenous imidazole[20] or various heterocycles[21,22] and the covalent incorporation of imidazole in replacement of ribozyme nucleobases[23,24] also implicate ribozyme functional groups as general acid/base catalysts.

The finding that the hairpin ribozyme does not require divalent metal cofactors for catalytic activity at high ionic strength[25−30] suggested early on that RNA nucleobases may participate directly in catalysis, possibly playing the role of general acid, general base, or both. Mutation studies have pinpointed particular nucleobases where functional group modification is particularly deleterious to hairpin ribozyme activity without affecting tertiary structure formation.[31−33] Analysis of the effect of nucleobase modification of G8 in the hairpin ribozyme has suggested that the N1 or possibly O^6 position of this residue is directly involved in hairpin ribozyme catalysis.[6,8,31] Furthermore, a synthetic hairpin ribozyme with a 1′-β-(4-imidazolyl)-riboside incorporated in place of G8 has recently been shown to be catalytically competent, further supporting the view that G8 assumes the role of the general base.[24] Crystal structure data are consistent with general base catalysis, showing the Watson−Crick face of G8 to be in close proximity to the nucleophilic 2′OH at the cleavage site.[4]

Nevertheless, other interpretations including stabilization of the developing negative charge in the transition state by G8, a purely structural role for G8, general acid catalysis, and the involvement of an active site water molecule in specific base catalysis could also be consistent with the existing data.[4,22,31,34] Given the possibility of kinetic ambiguity, multiple mechanisms that are kinetically indistinguishable can often be ascribed to pH−rate profile data and chemical rescue experiments.[35] The existing biochemical and biophysical data, while crucial in deciphering any enzymatic mechanism, still leave some room for interpretation.

Herein we have applied to the hairpin ribozyme an experiment directly analogous to that which identified His12 as the active site residue responsible for general base catalysis in RNaseA.[36] By replacing the 2′OH at the site of cleavage of the substrate with an electrophilic bromoacetamide group, we have created an affinity label that specifically alkylates G8 of the hairpin ribozyme (Scheme 1). Additional analysis strongly suggests that it is the N1 position of G8 that is alkylated. Implications of these data for the role of G8 in the catalytic mechanism of the hairpin ribozyme are discussed in the context of recent literature on the subject.

Scheme 1



Materials and Methods

Chemicals and Biochemicals: All chemicals and buffers were from Sigma-Aldrich. DNA oligonucleotides were synthesized by NAPS unit at UBC, and RNA oligonucleotides, by Dharmacon and Trilink Biotech. The T7 Mega Short Script kit (Ambion) was used for in vitro transcription of ribozyme RNA. The α- and γ-^{32}P-ATP were from Perkin-Elmer. Enzymes were from New England Biolabs and Fermantas.

Preparation of the Substrate Analogue: Typically, a solution of 20 nmol of substrate analogue **1** (Scheme 2) dissolved in 20 μL of 400 mM sodium borate buffer (pH 8) was combined with 20 μL of a 250 mM solution of **2** in dimethylformamide. The reaction was allowed to proceed for 5 min at room temperature, at which point further 40 μL portions each of borate buffer and **2** were added and the reaction continued a further 10 min. The reaction was then ethanol precipitated and vacuum-dried, and the process was repeated. Compound **2** was prepared fresh every week as previously described.[37]

Preparation of Ribozymes: Body labeled hairpin ribozymes were prepared by in vitro transcription[38] from synthetic DNA templates in the presence of α^{32}P-ATP. The wild type ribozyme sequence was: 5′-pppGGGAAAUAGAGAAGCGAACCAGAGAAACACACGACGUAAGUCGUGGUACAUUACCUGGUA-3′. Transcripts were purified by 10% denaturing PAGE, eluted with 1% $LiClO_4$/10 mM TrisHCl (pH 8) for 45 min at 65 °C, ethanol precipitated, and G-25 desalted prior to use. The mutant ribozyme with deoxy-7-deazaguanosine replacing G8 was prepared by ligation of a synthetic oligonucleotide containing the modification to a truncated ribozyme transcript comprising the remainder of the ribozyme sequence. This reaction was accomplished by annealing the two RNAs to be joined to a splint DNA and treating with T4 DNA ligase at 16 °C overnight.[39] The resulting full-length hairpin RNA was then gel purified and 5′-^{32}P labeled using T4 polynucleotide kinase (New England Biolabs) and γ^{32}P-ATP.

Alkylation Reactions: Alkylation reactions were performed in 50 mM sodium borate (for pH 8, 8.5, and 9) or sodium cacodylate (for pH 7.5) buffer with 50 mM $MgCl_2$ in the dark at room temperature (22−24 °C). The ribozyme concentration was 5 μM, and the concentration of the substrate analogue (a roughly 1:1 mixture of **1** and **3**) was 5.5 μM. Reactions were stopped with two volumes of 90% formamide/50 mM EDTA/0.01% xylene cyanol/0.01% bromophenol blue. Reaction products were resolved by 10% denaturing PAGE and quantified by phosphorimagery (Imagequant v 5.2).

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Footprinting of the Alkylation Site on the Ribozyme: Prior to bromoacetylation, **1** was 5′-phophorylated using unlabeled ATP and T4 polynucleotide kinase. The ribozyme was transcribed and then dephosphorylated by treatment with shrimp alkaline phosphatase. The sample was heat inactivated at 65° for 10 min, phenol chloroform extracted twice, and desalted over a G25 spin column prior to reaction with **3**. Unlabeled alkylated hairpin ribozyme was purified by 10% denaturing PAGE by UV shadowing and worked up as described for the ribozyme transcripts. The isolated alkylated ribozyme was then radiolabeled using γ^{32}P-ATP and T4 polynucleotide kinase. Only the 5′-end of the ribozyme is radiolabeled, as the 5′-end of the substrate strand was blocked by prior phosphorylation.

The 5′-end labeled alkylated ribozyme isolated as described above and pure 5′-end labeled ribozyme were each treated with 0.5% K_2CO_3 (room temperature, pH = 10.3) at 95 °C for 3 min in a volume of 20 μL to generate the hydrolysis ladders in Figure 3 (lanes 3 and 4). To generate the G specific cleavage ladder (lane 5 in Figure 3), the pure 5′-end labeled ribozyme was combined with 5 nmol of carrier RNA (16mer) in 10 μL of 1 mM TrisHCl (pH 7.5) and treated with 1 unit of RNase T_1 at 37 °C for 4 min. Reactions were terminated by adding 40 μL of 90% formamide/50 mM EDTA/0.01% xylene cyanol/0.01% bromophenol blue. The digestions were immediately analyzed on a 20% denaturing PAGE sequencing gel.

Nuclease Digestion/MALDI-TOF Mass Spectrometry: Approximately 2 nmol of alkylated hairpin ribozyme were prepared and purified by denaturing PAGE as described above. Following elution from the gel, the sample was ethanol precipitated, resuspended in water, and G-25 desalted. Digestion of the alkylated ribozyme was then carried out by adding 200 units each of RNase T_1 and *E. coli* RNase I and incubating the sample at 37 °C for 2 h. The sample was then further desalted by incubating the sample at room temperature for 1 h with a small portion of cation exchange beads (Bio-Rad AG50W-X8 resin, NH_4^+ form). The MALDI-TOF matrix used consisted of an 8:2 mixture of a 0.5 M solution of trihydroxyacetophenone in ethanol and a 2.4% aqueous solution of ammonium citrate. The matrix was combined in a 1:1 ratio with the aqueous digestion sample on the MALDI-TOF target and allowed to air-dry at room temperature. MALDI-TOF spectra were recorded on a Bruker Biflex II instrument in reflexive negative ion mode.

Results

The reactive substrate analogue **3** bearing the 2′-bromoacetamide group at the cleavage site was prepared by acylation of a 2′-amino precursor substrate analogue **1** with the *N*-hydroxysuccinimidyl ester of bromoacetate **2** (Scheme 2) (exocylic nucleobase amines do not react under these conditions).[40] The most successful conditions for bromoacetylation followed those employed in the bromoacetylation of 5′-amino groups.[37]

Reaction of the 2′-bromoacetamide substrate analogue with the trans-acting hairpin ribozyme construct yields a significant amount of a higher molecular weight species. The conditions found to provide the highest alkylation yield were a slight excess of substrate analogue over hairpin ribozyme in buffered 50 mM Mg^{2+} at room temperature (the effect of pH is discussed below). The significantly slower rate of the alkylation reaction compared to the cleavage reaction parallels a similar result for RNaseA: the ratio of the alkylation rate constant[36] to the cleavage rate constant[41] (k_{alk}/k_{cat}) is 4.1×10^{-6} for RNaseA and 0.013 for the hairpin ribozyme. The rates of cleavage and alkylation for both the hairpin ribozyme and RNase A are presented for comparison in Table 1.

Scheme 2

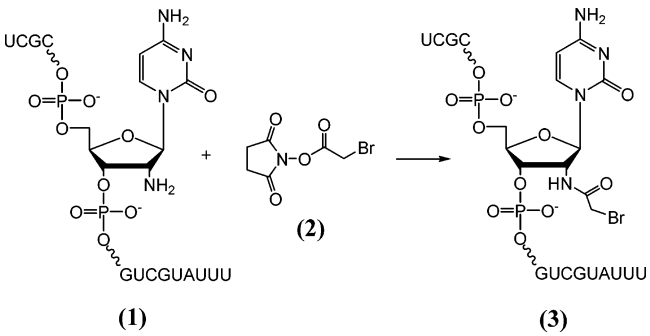

Table 1. Comparison of Substrate Cleavage and Alkylation Rate Constants for Wild type Hairpin Ribozyme and RNase A

	hairpin	RNase A
cleavage rate constant (k_{cat})	0.03 min^{-1} [a]	1.4×10^3 s^{-1} [b]
alkylation rate constant (k_{alk})	4×10^{-4} min^{-1} [c]	5.8×10^{-3} s^{-1} [d]
k_{alk}/k_{cat}	0.013	4.1×10^{-6}

[a] Data not shown. [b] Data for cleavage of UpA substrate from ref 40. [c] This work. [d] Data for alkylation with 2′-bromoacetamido-2′-deoxyuridine from ref 35.

The maximum fraction of ribozyme that could be alkylated was ~50% (for the G8A mutant, Figure 4A). The ribozyme is not completely alkylated for at least two reasons. (1) The unreactive precursor 2′-amino substrate analogue could not be separated from the reactive bromoacetylated substrate analogue before reaction with the ribozyme. Consequently, the precursor serves as a potent competitive inhibitor of the crosslinking reaction. This is demonstrated in Figure 4A where addition of a stoichiometric amount of the 2′-amino substrate analogue was found to almost completely inhibit alkylation activity. (2) The reactive bromoacetamide group on **3** is almost completely hydrolyzed to the hydroxyacetamide in buffer alone over the time course of the reaction (~60 h) as judged by mass spectrometric MALDI-TOF analysis (data not shown).

In order to identify the alkylated residue, the alkylated ribozyme (5′-^{32}P labeled) was purified by denaturing polyacrylamide gel electrophoresis (PAGE). It was then subjected to treatment with base in order to identify the alkylated residue by foot printing analysis. As demonstrated by the dramatic increase in molecular weight at G8 in the base hydrolysis ladder (Figure 2, lane 3), G8 of the hairpin ribozyme is specifically alkylated. Alkylation of G was also confirmed by MALDI-TOF mass spectrometric analysis of an RNase T_1/RNase I digest of the alkylated ribozyme. The minimal digestion fragment which proves alkylation at a G is shown in Figure 3. Note that two nucleotides are necessarily derived from the substrate analogue strand because the 2′-acetaminde group blocks RNase action upon the neighboring phosphodiester linkage.

The correlation of alkylation with hairpin cleavage activity was then examined with regards to several conditions including competition with a nonhydrolyzable substrate analogue, the presence or absence of Mg^{2+}, and in the context of mutant ribozymes known to perturb cleavage activity (all in Figure 4A). To show that the alkylation reaction is directed by substrate binding, a stoichiometric quantity of unreactive (2′NH_2) substrate analogue was found to compete with the affinity label (2′BrAcNH−) and essentially inhibit alkylation. Under buffer

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conditions favoring substrate hybridization as well as general secondary structures (50 mM sodium cacodylate pH 7.5) but lacking the Mg^{2+} necessary for tertiary structure formation and maximal catalytic activity, alkylation was almost completely abolished. Thus, alkylation of G8 occurs only when the ribozyme is folded in a catalytically competent conformation in the presence of Mg^{2+}.

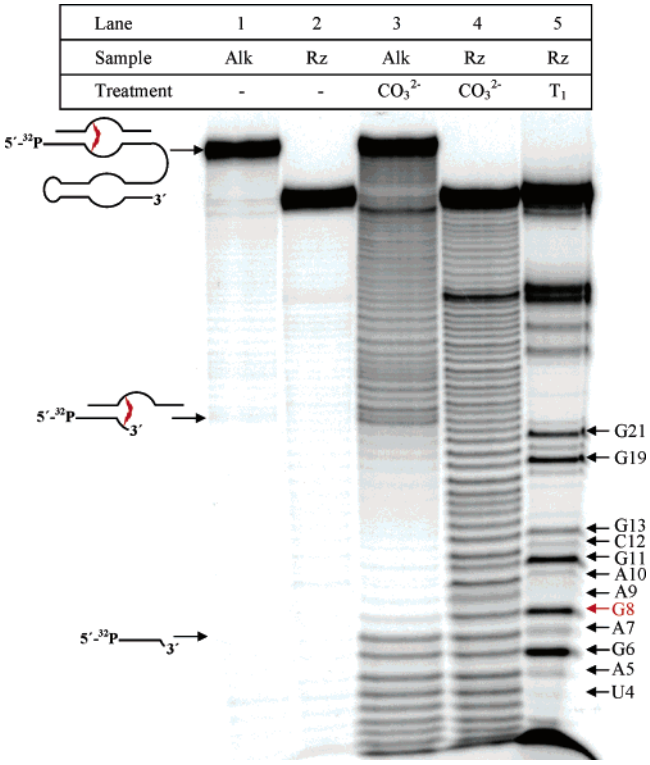

Figure 2. Footprinting analysis reveals the site of hairpin ribozyme alkylation by the bromoacetamide substrate analogue (Alk = alkylated hairpin ribozyme; Rz = hairpin ribozyme). 5′-^{32}P labeled fragments were separated on a 20% (19:1 monomer/bis) PAGE/7M urea sequencing gel and autoradiographed. Lane 1: purified alkylated hairpin ribozyme. Lane 2: purified hairpin ribozyme. Lane 3: alkylated hairpin ribozyme treated with 0.5% K_2CO_3. Lane 4: hairpin ribozyme treated with 0.5% K_2CO_3. Lane 5: hairpin ribozyme G ladder generated by RNase T_1 treatment. The ribozyme sequence as determined from the RNase T_1 G sequencing ladder (Lane 5) is depicted at right. The discontinuity in the base hydrolysis ladder for the alkylated ribozyme clearly indicates that the site of alkylation on the ribozyme is at G8. Some hydrolysis of the alkylation linkage during K_2CO_3 treatment is evident as background bands appear above G8 in lane 3.

We asked whether alkylation reflected specific aspects of catalysis or was simply an adventitious consequence of placing an electrophile in the vicinity of G8, as guanine is readily alkylated nonspecifically. To that end, we sought to determine which position of the guanine nucleobase was the site of alkylation. Generally, N7 is taken to be the most nucleophilic position of guanine.[42,43] However, if alkylation had occurred on N7, it would reflect a nonspecific alkylation event that in this case would be entirely unrelated to general base catalysis for the following two reasons: (1) the N7 position is unlikely to perform general base catalysis given its low pK_a, and (2) its distal position in the crystal structure with respect to the 2′OH makes it an unlikely participant in catalysis.[4] In order to

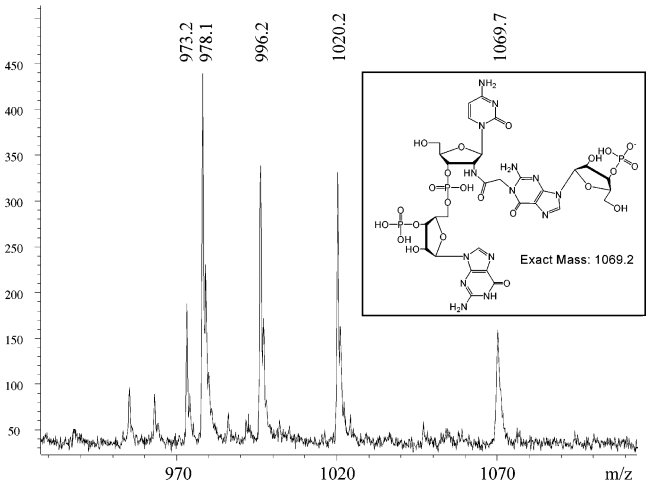

Figure 3. A portion of the MADLI-TOF mass spectrum obtained after digestion of the alkylated ribozyme with a mixture of RNase T_1 and *e. coli* RNase I. The data were acquired in reflexive negative ion mode using trihydroxyacetophenone/ammonium citrate as a matrix. The peaks are assigned as follows: CpUpGp (predicted *m/z*: 973.1; observed *m/z*: 973.2), CpApG>p, where >p refers to the 3′ cyclic phosphate (predicted *m/z*: 978.1; observed *m/z*: 978.1), CpApGp (predicted *m/z*: 996.1; observed *m/z*: 996.2), ApApGp (predicted *m/z*: 1020.2; observed *m/z*: 1020.2). The digestion fragment corresponding to G alkylation is shown along with its predicted mass.

definitively exclude N7 alkylation, an active mutant ribozyme was synthesized where 7-deaza-deoxyguanosine replaced G8.[31] It showed very similar alkylation activity to the wildtype ribozyme (Figure 4A), showing convincingly that N7 is not being alkylated. Alkylation at O^6 of G8 was also considered. Given that O^6-alklyated guanosines exhibit a characteristic fluorescent emission at 420 nm,[43] the alkylated product (~1 nmol) was PAGE purified, G25 desalted, and excited in the range 270−340 nm. No emission could be detected in the range 350−500 nm (data not shown).

Both the rate and extent of G8 alkylation increased modestly with increasing pH from 7.5 to 9 (see Figure 4B and Table 2), in contrast to the very flat pH−rate profile from pH 6−9 for the hairpin cleavage reaction.[5,31] This is to be expected because alkylation depends only on nucleophile deprotonation, whereas cleavage activity likely depends on balancing general base and general acid activity,[5] the latter being diminished at high pH. The rather shallow pH-dependence could have several explanations that are discussed in the next section (*vide infra*).

A G8A mutant, which shows significant catalytic impairment,[31] showed higher alkylation activity than the wild type. Not unexpectedly, this reflects a synergy of both structural positioning and the general nucleophilicity of adenosine. To begin with, free adenosine is readily alkylated at either N1 or N3 and to a lesser extent at N7,[42,44] all of which are deprotonated at neutral pH (pK_a of the conjugate acid of N1 is 3.7[11]). Apart from N7 alkylation, which has already been excluded in this case with the G8(7-deaza-dG) mutant, the general reactivity of adenine dictates alkylation on either N1 or N3 (the exocyclic N^6 of adenosine is not susceptible to alkylation).[42,44] Furthermore, alkylation of the G8A mutant showed no pH dependence (pH 7.5−9), again consistent with alkylation of fully deprotonated N1 (or N3) of adenine (data not shown). From a structural perspective, the crystallographic data of the hairpin ribozyme

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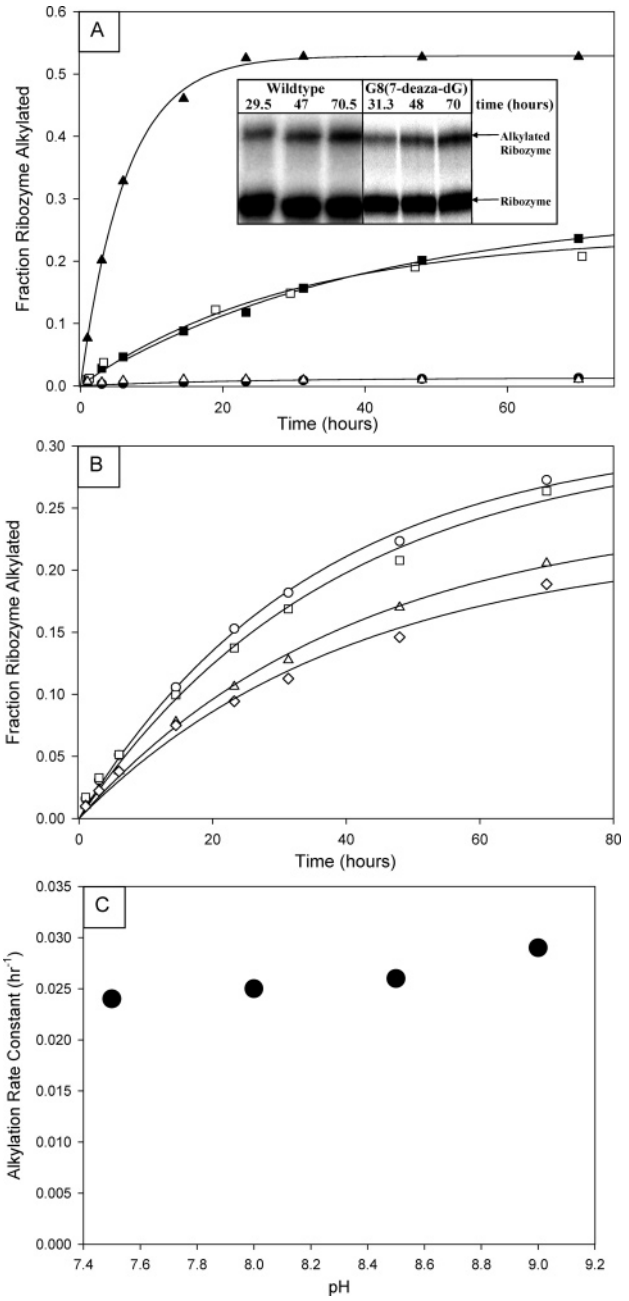

Figure 4. (A) Time course plots for hairpin ribozyme alkylation with the reactive bromoacetamide substrate analogue for the wildtype ribozyme (■), the G8A mutant (▲), the G8(7-deaza-deoxyguanosine) mutant (□), the wild type ribozyme in the absence of Mg^{2+} (△), and the wild type ribozyme in the presence of a stoichiometric amount of unreactive 2′-amino substrate analogue (●). In all cases the reaction was carried out in 50 mM sodium cacodylate pH 7.5 at room temperature in the dark with 5 μM ribozyme and 5.5 μM substrate analogue. Except for the reaction in the absence of Mg^{2+}, all reactions contained 50 mM $MgCl_2$. Reaction time points were quenched in two volumes of 90% formamide/50 mM EDTA/0.01% xylene cyanole/0.01% bromophenol blue and resolved by 10% PAGE (29:1 monomer/bis) containing 7 M urea. Gels were exposed to storage phosphor screens, and data were quantified using Imagequant v5.2. Inset: representative alkylation of the wild type and G8(7-deaza-deoxyguanosine) ribozymes visualized by autoradiography of PAGE resolved samples. (B) Time course plots for wild type hairpin ribozyme alkylation at varying pH values. The reaction conditions were: 50 mM sodium cacodylate pH 7.5 (◇), 50 mM sodium borate pH 8.0 (△), 50 mM sodium borate pH 8.5 (□), and 50 mM sodium borate pH 9 (○). All reactions contained 50 mM $MgCl_2$ and were quenched and analyzed as described for part A. (C) Wild type hairpin ribozyme alkylation rate constant plotted as a function of reaction buffer pH.

itself suggest that N1 is the site alkylation and not N3.[4] Thus, N1 reflects the only chemically consistent position for alkylation between the wild type G8 and the G8A mutant.

Table 2. Rate Constants and Extents of Reaction for the Hairpin Ribozyme Alkylation Reactions[a]

ribozyme/conditions	alkylation rate constant, k_{alk} (hr^{-1})	maximum fraction of ribozyme alkylated
wild type:		
pH 7.5	0.024	0.22
pH 8.0	0.025	0.25
pH 8.5	0.026	0.31
pH 9.0	0.029	0.31
G8A pH 7.5	0.16	0.53
G8(7-deaza-G) pH 7.5	0.034	0.24

[a] Rate constants given in Table 2 correspond to the data given in Figure 4A−C.

Discussion and Conclusions

The alkylation strategy described herein functions specifically under conditions that support the normal hairpin ribozyme cleavage reaction. This suggests that the alkylation reaction is a direct consequence of the proper placement of the functional groups responsible for hairpin ribozyme catalysis. The finding that G8 is specifically alkylated demonstrates that, at physiological pH in the folded hairpin structure, G8 is well positioned near the 2′OH which attacks the phosphate in the first step of ribophosphodiester hydrolysis. The data presented herein confirm in solution the crystal structure data which place N1 of G8 in close proximity to the cleavage site 2′OH, as an active participant in catalysis.[4] Nevertheless, with any affinity label, one must entertain a *caveat* as to whether the label is so different structurally that the labeling it affords is not reflective of catalytic competence, but rather general proximity effects, or perturbed reactivity in an active site that is altered upon binding the label. Although the data from the G8(7-deaza-G) and G8A mutants exclude nonspecific N7 alkylation, we must be cautious in drawing mechanistic conclusions regarding catalysis when the same could be simply attributed to fortuitous proximity effects in the alkylation at N1 of G8.

Given the strong analogy to the case of RNaseA, our results suggest that G8 may be involved in some form of general base catalysis. However, due the size of the bromoacetamide probe, participation of a bridging water molecule in the active site mediating proton transfer between the substrate 2′OH and G8 cannot be excluded. This interpretation is supported by recent crystal structures in which a water molecule was observed in the active site.[34] By contrast, it should be noted that there is no evidence of water mediated general base catalysis in RNaseA, the enzyme to which the analogous alkylation experiment was successfully applied.[36] Interestingly, the higher k_{alk}/k_{cat} for the hairpin ribozyme compared to RNaseA (Table 1) might be attributable to (1) a different general base mechanism that is mediated by a water molecule and/or (2) differences in the overall flexibility of the active sites (catalytic RNAs being considerably more flexible than proteins[12]) where the RNA scaffold of the hairpin is more accommodating than the protein scaffold of RNaseA.

The pH−rate data presented here (Figure 4B and C, and Table 2) indicate that the rate and extent of the alkylation reaction are enhanced only slightly at higher pH over the range of pH

7.5 to 9. The slight increase in alkylation rate at pH 9 compared to pH 7.5 is much less pronounced than would be expected for G8 with a pK_a of 9, since the anionic fraction of G8 and, thus, the value of k_{alk} would be expected to increase markedly as the pH approached 9. The rate independence of alkylation with respect to increasing pH does not seem consistent with a pK_a value of ~9 for G8, a reasonable assertion were G8 to serve as a general base.[5] It is always possible that the affinity label perturbs the active site structure such that the N1 proton of G8 may, in this case, have a higher pK_a than what has been suggested by the pH−rate profile for ribophosphodiester hydrolysis in which G8 acts as the general base (p$K_a \approx 9$).[5] Alternatively, an anticipated log−linear relation between the alkylation rate constant and pH reflecting G8 deprotonation could be offset by concomitant pH dependent conformational (denaturing) changes in the ribozyme that would have a negative effect on k_{alk}.

Bearing this in mind, the pH independence of the alkylation reaction would suggest the absence of a titratable proton at N1 of G8 with a pK_a within the pH range examined. Thus, G8 is either entirely deprotonated (p$K_a < 5.5$) or almost entirely protonated (p$K_a > 10.5$) in the pH range investigated. If G8 is entirely deprotonated at N1 in the docked state, it exists as an enolate anion, the conjugate acid of which would be expected to have a low pK_a.[45] An anionic state would also be consistent with both the flat pH rate profile and role for G8 as the general base. The enolate tautomer is also consistent with alkylation of the G8A mutant that was examined to test the regioselectivity of alkylation.

Stabilizition of the transition state by protonated N1 of G8 has also been proposed based on hydrogen bonding observed between G8 and the substrate 2′OH in the transition state analogue crystal structure.[4] Furthermore, synthetic mutants presenting heterocycles with lower N1 pK_a values, as well as G8-abasic mutants that are rescued by exogenous addition of similar heterocycles, show drastically altered pH−rate profiles, with lower activity at lower pH values than that observed for the native hairpin ribozyme.[22,31] These findings logically support the hypothesis that N1 of G8 must be protonated for activity, possibly stabilizing the negative charge on the 2′-oxygen in the transition state. However, as noted by Bevilacqua, loss of general acid activity could explain the narrowed pH−rate profiles for low pK_a G8 analogues such as diaminopurine.[5] The nearly flat pH−rate profile for the alkylation reaction could also be consistent with the transition state stabilization model[22] where G8 must be protonated for cleavage activity to stabilize an incipient negative charge on the 2′OH.[31] If the role of G8 is restricted to transition state stabilization, then this affinity labeling simply reveals G8's proximity to the 2′OH while some cryptic functionality that serves as the general base remains entirely undetected.

In summary, this work represents the first example of a mechanism-based affinity labeling approach to studying ribozymes. Although the current data do not seem to definitively distinguish between general base or transition state stabilization roles for G8, it is clear that the Watson−Crick face of G8 is poised to actively participate in catalysis. In a more general sense, these results highlight the mechanistic commonalities that can be shared by ribozymes and protein enzymes despite the apparent lack of useful functional groups in RNA. This approach may also find use in probing the active sites of other ribozymes and metal-independent DNAzymes with synthetic functionalities mimicking RNaseA,[46,47] as well as the ribosome. This methodology is also being explored for use in RNA profiling by analogy to proteomic profiling, for the discovery of new ribozymes.

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Supporting Information Available: Autoradiography images for alkylation of bodylabeled wild type and mutant hairpin ribozymes; full MALDI-TOF spectrum of RNase T_1/I digest of the alkylated catalyst and table of MALDI-TOF peak assignments. This material is available free of charge via the Internet at http://pubs.acs.org.

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(45) To the best of our knowledge, there is no good experimental data on the pK_a of the conjugate acid of a G-enolate; however if the keto-enol ratio of the free nucleoside reflects the difference in the respective pK_a's at N1 or O^6, then the pK_a of the G8-enolate will be low, although in the case of the hairpin ribozyme this value could be perturbed upwards.

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