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Steric Interference Modification of the Hammerhead Ribozyme

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Summary

Although the structure of the hammerhead ribozyme is well characterized, many questions remain about its catalytic mechanism. Extensive evidence suggests the necessity of a conformational change en route to the transition state. We report a steric interference modification approach for investigating this change. By placing large 2′ modifications at residues insensitive to structurally conservative 2′-deoxy modifications, we hoped to discover structural effects distal to the site of modification. Of twenty residues tested, six were identified where the addition of 2′ bulk inhibits cleavage, even though these bulky modifications could be accommodated in the crystal structure without steric clash. It is proposed that these 2′-modifications inhibit cleavage by preventing formation of the alternate, active conformation. Since these 2′ effects are present in both domain I and domain II of the hammerhead, the entire catalytic core must undergo conformational changes during catalysis.

Introduction

In the presence of modest concentrations of divalent metal ions, the hammerhead ribozyme autolytically increases the cleavage rate of a specific phosphodiester bond within its sequence by nearly 10^6-fold relative to the uncatalyzed reaction [1, 2]. An important question to address is how the hammerhead achieves this significant rate enhancement. Among several possible contributors to the rate enhancement are general base or acid catalysis [3, 4], the stabilization of the transition state by a specific divalent metal [1], or proper conformational alignment of the scissile phosphate [5]. The folded RNA structure undoubtedly facilitates each of these factors by bringing the reactive groups into proximity. Although several crystal structures of the hammerhead are available [6, 7], numerous disagreements between these structures and the biochemical data identifying essential functional groups suggest that these structures may not represent the active conformation [8]. Rather, one or more conformational changes occur en route to the transition state. Understanding the structure of the proposed active conformation would presumably reveal how the essential functional groups interact in the transition state and explain the observed rate enhancement.

One part of the crystal structure expected to change during catalysis is in the neighborhood of the cleavage site. Since inversion of steriochemical configuration occurs about the scissile phosphate [9], the 2′-oxygen of C_{17} most likely attacks the phosphate in line with the leaving phosphorous-5′-oxygen bond. Because the structure near the cleavage site is not near an in line conformation, it was suggested that the phosphodiester backbone at the cleavage site must rearrange prior to catalysis [6]. Indeed, the crystal structures of several proposed reaction intermediates displayed several small changes in domain I relative to the initial structure, including rotation of C_{17} around the scissile phosphate nearer to an in-line arrangement and repositioning of the bases in the CUGA loop [4, 10, 11]. Beyond these changes near the cleavage site, however, the structure of the remainder of the hammerhead was relatively unchanged, perhaps due to constraints imposed by the crystal lattice. Although it remains to be shown that these structures represent genuine reaction intermediates, they present a model for hammerhead cleavage in which the transition state is reached by relatively modest conformational changes near the cleavage site.

A second model suggests that a more extensive hammerhead conformational change occurs prior to the transition state. This model is based on biochemical experiments that suggest that a divalent metal ion coordinated to phosphate 9 in domain II distal to the cleavage site is an active participant in the transition state [12]. When this site is occupied by a thiophilic cadmium ion, it can "rescue" the deleterious effect of a monophosphorothioate modification at the cleavage site more than 20 Å away [13]. The sensitivity of this rescue to perturbations in the domain II site implies that the two sites coordinate the same metal in the transition state, suggesting an extensive structural rearrangement during catalysis that involves docking of domains I and II. This extensive conformational change would presumably also explain other structure-function inconsistencies observed in both domains of the hammerhead.

Here, we report a different type of RNA functional group analysis designed to give additional insight into the nature of the conformational changes involved in hammerhead catalysis. Whatever the nature of the conformational change, certain regions of the structure are expected to be more proximal in the active conformation than they are in the crystal structure. If a sterically bulky group were placed in such a region, close approach would be inhibited and the cleavage rate reduced. The cleavage rate should be unaffected by the introduction of bulky groups at regions in the hammerhead which

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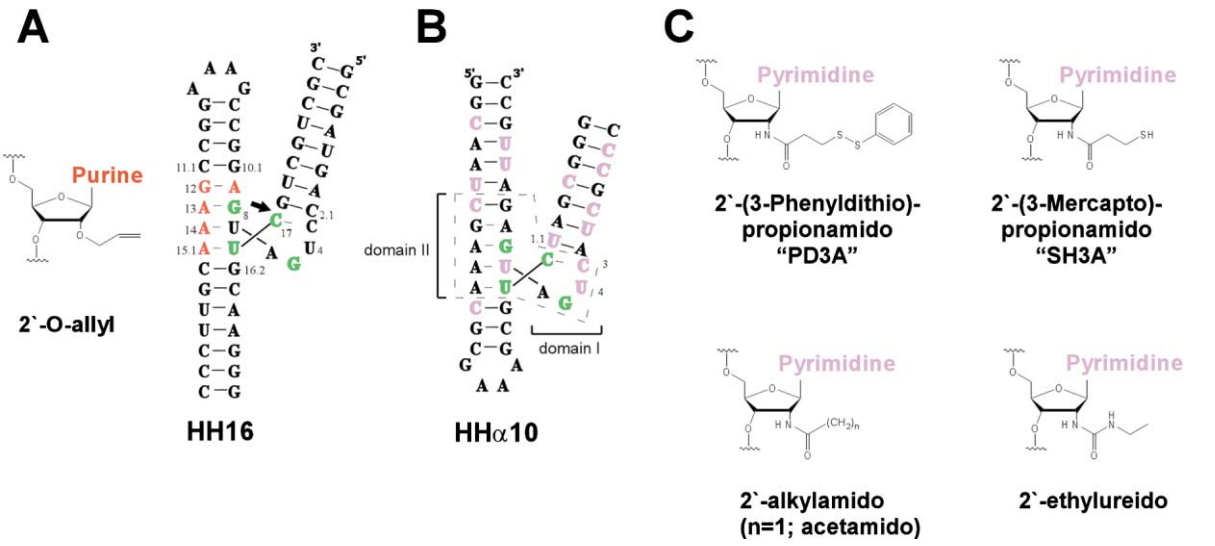

Figure 1. 2′-Modifications that Were Tested in the Hammerhead

(A) The 2′-O-allyl modification was incorporated at purine positions shown in red in hammerhead 16 [20]. Green signifies those residues where a 2′-deoxy modification decreases the cleavage rate by at least 10-fold [14–19], which were not tested. Residues are numbered as previously described [41].

(B) Residues in HHα10 [21] where a single 2′-amino-uridine or cytidine was incorporated are shown in purple. The catalytic core is outlined with a dashed line, and the two structural domains are noted.

(C) Pyrimidine 2′-modifications. Reduction of 2′-(3-phenyldithio)-propionamido yields 2′-(3-mercapto)-propionamido.

are not closely packed in the active conformation. Thus, by placing bulky groups at various parts throughout the hammerhead, information about the regions of the hammerhead that undergo conformational change is obtained. The results show that bulky modifications in both domain I and domain II inhibit catalysis and therefore suggest a relatively large conformational change. These results also suggest the generality of using steric interference modification to reveal conformational properties of RNA molecules.

Results

Modification of the 2′ Position

The 2′ position of each residue was chosen for the attachment of steric bulk for two reasons. First, there are only four residues in the hammerhead (G_5, G_8, $U_{16.1}$, and C_{17}) where the introduction of a single 2′-deoxy nucleotide inhibits cleavage by 10-fold or more [14–19]. Although the reasons for all of these specific deoxy effects are not clear from the crystal structure, presumably the 2′-hydroxyl group is required to maintain the active conformation. Thus, the remaining 2′-hydroxyls are not essential for catalysis and are potential sites for modification. Second, the crystal structure suggests that nearly all the 2′-hydroxyls protrude into the solvent and thus appear able to accommodate significant steric bulk without van der Waals overlap with other parts of the molecule. Therefore, steric bulk was introduced at twenty 2′ positions throughout the hammerhead which have no 2′-hydroxyl requirement.

Two types of large 2′ modifications were incorporated into two different hammerhead ribozymes using standard phosphoramidite chemistry. For the purine residues tested, a single 2′-O-allyl or 2′-O-methyl adenosine or guanosine residue was incorporated into the well-studied HH16 (Figure 1A) [20]. For each of the pyrimidine residues tested, versions of HHα10 (Figure 1B) [21] containing a single 2′-amino cytosine or uracil residue which were used in a previous crosslinking study [22] were used as a starting point for introducing steric bulk. Both HH16 and HHα10 are kinetically well-behaved hammerheads, formed by long intermolecular helices, and both show similar cleavage rates at saturation (k_2). Because substrate dissociation for both hammerheads is very slow compared to the rate of cleavage ($k_2 >> k_{-1}$) [20, 21], the rate at subsaturating substrate concentrations is dependent only on the rate of binding (k_1) [23], such that effects of modification on substrate dissociation (k_{-1}) would not be observed.

To incorporate steric bulk at the 2′-position of pyrimidine residues, 3-(phenyldithio)-propionic acid, a disulfide crosslinking reagent [22], was conjugated to the 2′-amine of the deprotected, purified HHα10 variants using the water-soluble carbodiimide EDC to give a 2′-(3-phenyldithio)-propionamido derivative (PD3A[1]; Figure 1C). Because of its higher nucleophilicity, the 2′-amine is modified selectively over base amines [24]. For the ribozyme strand, this oligonucleotide conjugate was reduced with dithiothreitol to yield the 2′-(3-mercapto)-propionamido derivative (SH3A; Figure 1C). After testing the modified hammerheads for cleavage (see below), those 2′-amine-containing hammerheads that showed an effect when modified with these large derivatives were subsequently modified with a series of smaller carboxylic acids in which the number of alkyl carbons was varied from one (acetic acid) to three (butyric acid; Figure 1C). The identity of all 2′-modified hammerheads

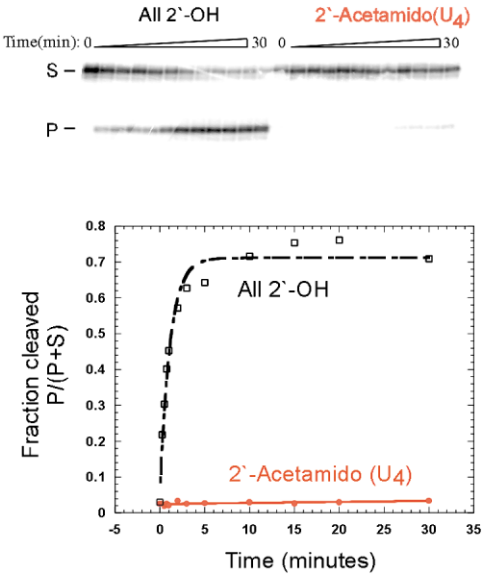

Figure 2. Cleavage of Modified Hammerheads

Denaturing polyacrylamide gel electrophoresis of a cleavage reaction. The fraction cleaved, P/(P+S), is plotted as a function of time. The curve is fit to the equation $(P/(P+S))_t = (P/(P+S))_0 + (P/(P+S))_\infty[1-\exp(-k_2 t)]$ to yield k_2.

was verified using MALDI-TOF mass spectrometry. The yield of amidation reactions generally exceeded 95%.

Cleavage Properties of 2′-Modified Hammerheads

The rate of cleavage was measured for each of the 2′-modified hammerheads in 100 mM HEPES (pH 7.5) at 25°C [23]. Cleavage rates were determined in the presence of 10 mM $MgCl_2$ using trace-labeled substrate and saturating ribozyme by following the conversion of the substrate to a smaller product strand, which can be fractionated on denaturing PAGE (Figure 2A). Quantitating the fraction cleaved as a function of time yields a plot to which a single-exponential function can be fit from which k_{obs} is derived (Figure 2B). Because the ribozyme is in excess, all of the labeled substrate is bound when the reaction is initiated. Thus, the substrate association and product dissociation steps are avoided, and k_{obs} is equal to the sum of the cleavage and ligation rates, k_2 and k_{-2}. In all cases, the extent of cleavage was greater than 90%, indicating that the ligation rate is slow, as typically observed for most hammerheads [25]. Thus, k_{obs} primarily reflects k_2, the rate of the chemical cleavage step. For each position where a 2′-alkylamido effect was observed, the cleavage rate of the unmodified 2′-amino-containing hammerhead was measured as well.

The effect of 2′-O-allyl purine modification on cleavage was measured for five different purines within the catalytic core (Table 1). A 2′-O-allyl modification at A_9 or A_{13} does not inhibit cleavage. In contrast, a 2′-O-allyl-modification at G_{12}, A_{14}, or $A_{15.1}$ inhibits the cleavage rate by 20-, 500-, and 200-fold, respectively. Interestingly, A_{13}, which shows no effect, is positioned between G_{12} and A_{14}, which both show significant effects.

Table 1. Cleavage Rates for 2′-O-Allyl-Purine-Modified Hammerheads

Position	2′-Modification	k_2 (relative)
A_9	O-allyl	1.05 ± 0.2
G_{12}	*O-allyl*	*0.048 ± 0.04*
A_{13}	O-allyl	1.58 ± 0.27
A_{14}	*O-allyl*	*0.0018 ± 0.0018*
$A_{15.1}$	*O-allyl*	*0.0053 ± 0.0018*

The effect of a 2′-alkylamido modification on cleavage was measured for fifteen different pyrimidine residues within the hammerhead (Table 2). Twelve of these residues are unaffected ($k_{rel} > 0.5$) by the addition of a large 2′-alkylamido modification. These include many positions throughout stems I and II as well as U7 and C11.1 within the catalytic core. At only three of the fifteen pyrimidine residues tested, $U_{1.1}$, C_3, and U_4, a 2′-alkylamido modification inhibits cleavage, by 8-, 5-, and 32-fold, respectively (Table 2). At each of these positions, the corresponding 2′-amino-modified hammerheads cleave nearly as well as the all 2′-OH control, suggesting that the bulk added onto the 2′-amine is responsible for the reduced rate.

To screen for size dependence at each of the positions where a 2′ effect is observed, several smaller modifications were introduced. For the purine residues where a 2′-O-allyl effect is observed, the effect of a 2′-O-methyl modification was also measured. At G_{12} and $A_{15.1}$, the 2′-O-methyl modification inhibits cleavage the same as the 2′-O-allyl modification. However, a 2′-O-methyl at A_{14} has relatively little effect on the rate of cleavage. Thus, the size of the modification affects the rate of cleavage at this position.

To test the size dependence of the 2′-alkylamido pyrimidine effects, the formamido, acetamido, propionamido, and butyramido modifications were incorporated at the 2′-positions of C_3, U_4, and $U_{1.1}$ and their effects on cleavage measured (Table 3). At each of these three positions, changing the size of the alkyl group has little effect on the cleavage rate. In all cases, the smallest 2′-formamido modification is sufficient to inhibit cleavage.

Hendrix and coworkers reported modest destabilization of RNA helices by the 2′-acetamido modification, perhaps because of an induced change in sugar puckering or other local structural defect [26]. Thus, it was possible that the observed 2′-acetamido effects in the hammerhead were due to alteration of the sugar pucker or ribose structure, rather than interference by the steric bulk. To address this possibility, we tested the effect of a 2′-ethylureido modification (Figure 1C), which is similar in size to 2′-acetamido but does not affect helix stability or structural behavior [24]. To incorporate a 2′-ethylureido modification, ethylisocyanate was conjugated to the 2′-amine of $U_{1.1}$, C_3, or U_4 of HHα10 as previously described [24], and the cleavage rate of the resultant hammerheads assayed. As shown in Table 3, a 2′-ethylureido modification at $U_{1.1}$, C_3, and U_4 inhibits cleavage similarly to the respective 2′-acetamido modifications. This suggests that the 2′-alkylamido effects are not specific to the amide linkage and are not inherently linked to helical or structural destabilization.

Table 2. Cleavage Rates for 2′-Alkylamido-Pyrimidine-Modified Hammerheads

Position	2′-modification	k_2 (relative; min^{-1})
All-2′-OH	—	1.0
$C_{2.6}$	SH3A	0.841 ± 0.10
$C_{2.5}$	SH3A	0.98 ± 0.049
$C_{2.3}$	SH3A	0.69 ± 0.15
$U_{2.2}$	SH3A	0.35 ± 0.007
C_3	*butyramido*	*0.201 ± 0.094*
U_4	*SH3A*	*0.031 ± 0.007*
U_7	SH3A	0.89 ± 0.147
$U_{10.3}$	SH3A	1.05 ± 0.12
$U_{10.4}$	SH3A	0.76 ± 0.12
$C_{11.5}$	PD3A	0.87 ± 0.12
$U_{11.2}$	PD3A	0.38 ± 0.21
$C_{11.1}$	butyramido	0.51 ± 0.042
$C_{15.2}$	butryamido	0.38 ± 0.17
$U_{1.1}$	*PD3A*	*0.123 ± 0.033*
$C_{1.4}$	PD3A	1.5 ± 0.64

The hammerhead ribozyme is known to cleave in high concentrations of monovalent ions as well as in divalent ions [3, 27, 28]. It is conceivable that the mechanism of cleavage could be slightly different in the two ionic environments, possibly leading to a different interference pattern of the large 2′ modifications tested here. To address this possibility, the cleavage rates of several of the 2′-modified hammerheads were measured in the presence of 2 M or 4 M LiCl. For all of the 2′-modified hammerheads tested, the magnitude of inhibition was the same in LiCl as in $MgCl_2$ (data not shown).

Discussion

The common paradigm in structure-function analyses in both proteins and nucleic acids is to make modifications that are the same size or smaller than the residue being changed. It is commonly reasoned that the steric interference induced by a larger modification produces unwanted or ambiguous effects. Here, a complimentary approach was taken by introducing bulky functional groups at positions known to be insensitive to structurally conservative modifications. The goal was to discover structural constraints whose effects are more distal to the site of modification.

Table 3. Varying the Size of Inhibitory 2′ Modifications

Position	2′-Modification	k_2 (relative)
C_3	amino	0.485 ± 0.040
C_3	acetamido	0.222 ± 0.086
C_3	propionamido	0.095 ± 0.079
C_3	butyramido	0.163 ± 0.076
C_3	ethyl-ureido	0.318 ± 0.004
U_4	amino	0.295 ± 0.043
U_4	formamido	0.050 ± 0.007
U_4	acetamido	0.019 ± 0.002
U_4	propionamido	0.018 ± 0.001
U_4	butyramido	0.010 ± 0.004
U_4	ethyl-ureido	0.086 ± 0.004
$U_{1.1}$	amino	1.16 ± 0.014
$U_{1.1}$	formamido	0.134 ± 0.041
$U_{1.1}$	acetamido	0.039 ± 0.002
$U_{1.1}$	propionamido	0.116 ± 0.051
$U_{1.1}$	butryamido	0.041 ± 0.013
$U_{1.1}$	ethylureido	0.117 ± 0.016
G_{12}	O-methyl	0.15
G_{12}	O-allyl	0.048 ± 0.04
A_{13}	O-methyl	1.50
A_{13}	O-allyl	1.58 ± 0.27
A_{14}	O-methyl	0.75
A_{14}	*O-allyl*	*0.0018 ± 0.0018*
15.1	O-methyl	0.001
15.1	O-allyl	0.0053 ± 0.0018

Using this approach, we have introduced bulky modifications at 2′ positions throughout the hammerhead at sites where a 2′-deoxy modification has no effect. Most of these large modifications have no inhibitory effect on the rate of cleavage, implying that they do not affect the transition state structure of the hammerhead. However, three pyrimidine and three purine residues were identified, all in or adjacent to the conserved catalytic core, where the introduction of a sterically large 2′-modification does inhibit cleavage, suggesting destabilization of the transition state structure.

It is informative to compare these results with previous studies investigating the consequences of placing similar 2′ modifications within the hammerhead. Heidenreich and coworkers [29] also observed only a slight effect (k_{rel} = 0.3) of a 2′-amino modification at U4, U7, and G9. While screening for nuclease-resistant ribozymes, Paolella and coworkers [30] found that 2′-O-allyl U4, A6, G12, or A15.1 each individually caused a substantial decrease in cleavage efficiency, while 2′-O-allyl A12 and A13 only caused slight decreases in cleavage efficiency. While these studies were not highly quantitative, they confirm the results presented here. Finally, Hendrix and coworkers [31] showed that a single 2′-acetamido cytosine at C3 caused a slight decrease in the observed cleavage efficiency, consistent with the results presented here.

To understand the structural implications of adding bulk at each of these 2′ positions, the modifications were modeled into the crystal structure [7]. Using the Insight II molecular visualization program (Accelrys Inc.), either a 2′-acetamido or a 2′-O-allyl was modeled onto each pyrimidine or purine position that had been biochemically tested. At many of these positions, the 2′ oxygen was sufficiently close to other atoms in the hammerhead that in some orientations, the modification encountered van der Waals overlap with other atoms in the hammerhead. Therefore, multiple orientations of the modification were analyzed at each position. For the 2′-acetamido modification, two dihedral angles were varied: the angle around the 2′-C/2′-N bond (ϕ) and the rotation of the methyl group (ψ; Figure 3A). In all cases, the planarity of the amide moiety was maintained, and only the *trans* isomer was considered. Similarly, for the 2′-O-allyl modification, the angle around the 2′-C/2′-O bond (ϕ) and around the 2′-O/C_α bond (ω; Figure 3A) were allowed to vary. At 19 of the 20 positions examined, multiple different combinations of ϕ and ψ or ϕ and ω permitted accommodation into the structure without overlap. For instance, Figure 3B illustrates one of several ways the 2′-acetamido modification can be accommodated at residues C_3 and U_4 without overlap. It is important to note that the number of possible rotamers and the values of ϕ that permit steric accommodation varied among the residues, and that there is no correlation

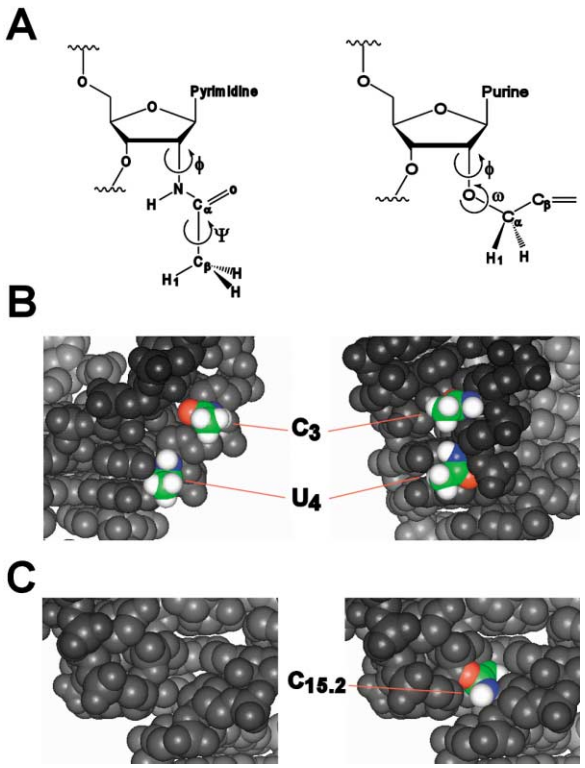

Figure 3. 2′-Modifications Modeled into the Crystal Structure [7]

(A) The 2′-acetamido and 2′-O-allyl modifications. The 2′-acetamido dihedral angles varied were ϕ (C3′-C2′-N2′-H1$_{N2'}$) and ψ (O-C$_\alpha$-C$_\beta$-H$_1$). Only the *trans* isomer of the amide was considered as shown. For the 2′-O-allyl modification, the angles ϕ (C3′-C2′-O2′-C$_\alpha$) and ω (C2′-O2′-C$_\alpha$-H1$_{C\alpha}$) were varied.

(B) 2′-acetamido-C$_3$ and U$_4$ can be accommodated into the crystal structure without van der Waals overlap. For the 2′-acetamido modification, carbon is shown in green, nitrogen in blue, oxygen in red, and hydrogen in white. Two different views demonstrate that there is no local steric interference induced by these modifications.

(C) 2′-acetamido C$_{15.2}$ cannot be accommodated in the structure. The left panel demonstrates that this region of the structure is sterically crowded. The right panel shows the 2′-acetamido modeled onto C$_{15.2}$, causing extensive van der Waals overlap with the surrounding residues.

between the number of possible rotamers or their particular angles and the biochemical activity. Indeed, while both 2′ modified C$_3$ and U$_4$ show substantial reduction in cleavage activity and U$_7$ does not, all three positions appear to accommodate the modification with equal ease. Interestingly, at only one position, C$_{15.2}$, were we unable to find an orientation of the 2′-acetamido that did not result in significant overlap. Figure 3C demonstrates that the 2′-position of C$_{15.2}$ is at the junction of the two structural domains where there is little room for the 2′-modification without steric clash. Thus, it is striking that the 2′-acetamido modification at this residue does not inhibit cleavage (Table 2).

Given this lack of correlation between the modeling exercise and the solution data, how could the bulky modifications be exerting their inhibitory effect? One possibility is that they alter the ribose sugar pucker to a conformation unfavorable for cleavage. Although the energetic difference between the C2′ endo and C3′ endo isomers for most 2′ derivatives is relatively small with free nucleotides [32], it is possible that a larger effect could be observed in the context of the folded hammerhead structure, even though none of the ribose conformations appear dramatically altered in the crystal structure. Indeed, it has been proposed that the 2′-amide moiety may destabilize RNA helices by disfavoring the 2′-endo sugar pucker that is typical of A-form helices [26]. Therefore, we tested the effect of a 2′-ethylureido modification on hammerhead cleavage, since this modification does not destabilize helical RNA and is not expected to affect sugar puckering [24]. For each residue where a 2′-alkylamido reduced the cleavage rate, the 2′-ethylureido group reduced cleavage a similar amount. This argues against a sugar-pucker explanation for the 2′-alkylamido affects shown here. Similarly, the purine 2′-effects are unlikely to reflect sugar pucker alteration, since the 2′-O-allyl modification actually stabilizes RNA duplexes [33].

Another possible explanation for the observed 2′ bulk-effects is that the modification is sufficiently hydrophobic to rearrange the local structure with a resultant effect on the cleavage rate. In fact, many of the modifications presented here involve the addition of one or more carbon atoms onto the 2′-position that should increase the hydrophobicity. However, at most of the sensitive sites, a variety of modifications with quite different hydrophobicities cause quite similar inhibitory effects. For instance, at C$_{1.1}$, the formamido, butyramido, ethylureido, and 3-mercapto-propionamido modifications all inhibit cleavage between 9- and 25-fold. Thus, it seems unlikely that the 2′-effects observed here are the result of hydrophobic destabilization of local hammerhead structure.

The energy associated with accommodating the modifications into the active hammerhead structure might also explain the observed 2′-effects. When the hammerhead is unfolded, it is expected that the 2′-modifications can more easily rotate around the 2′-C/2′-N (pyrimidine) or 2′-C/2′-O (purine) bond (ϕ) with modest energetic minima and maxima [34, 35]. Assembly of the hammerhead into the active structure will limit the set of rotational conformers that the 2′-modifications could assume with an accompanying entropic penalty. In addition, the active conformation may only be able to form using a high energy rotamer. The active conformation considered here would have to be in some way different from the crystal structure, since the modeling study discussed above indicated that many rotamers could be accommodated at nearly every 2′ position in the crystal structure, and there was no correlation between the number or type of rotamers that could be accommodated and the cleavage rate of the modified hammerhead. While the enthalpic and entropic cost of rotamer restriction about these bonds is generally relatively modest [36], it is conceivable that they could account for at least some of the observed decreases in activation energy. However, the fact that modifications of very different size and chemical composition have similar effects on the cleavage rate argues against rotational restriction being the major source of hammerhead inhibition. For example, the 2′ ureido and 2′ alkylamido modifications have a similar size and inhibit cleavage equally, despite the fact that the two groups should have different energies associated with each rotational state [37].

The most straightforward explanation for the decrease in hammerhead cleavage rate caused by the 2′ substituents is that for all possible rotamers, the added bulk causes a steric clash that prevents the active conformation from forming as efficiently. Since at nearly all positions, the inhibitory effect is observed when a single carbon is added to the 2′ oxygen, it is suggested that the six essential 2′ positions must closely approach a different part of the structure in the active conformation in such a way that none of the rotamers can be accommodated. The comparatively large (up to 1000-fold) effects of the bulky residues on the cleavage rate are also consistent with steric clash. In addition to numerous examples with small molecule substrates of enzymes, there are many examples where the steric clash caused by a single carbon atom can cause similarly large effects in the rate of protein enzymes that interact with nucleic acids [38].

Since the crystal structure can accommodate all six of the large 2′-modifications that inhibit cleavage, it must not represent the active hammerhead conformation whose formation is inhibited. Rather, the initially formed ribozyme-substrate complex, RS, must be in equilibrium with an alternate active conformation, RS*, which must form prior to cleavage:

$$RS \overset{K_{eq}}{\rightleftharpoons} RS^* \xrightarrow{\text{chemical step}} RP$$

The conformation of RS is probably best described by the crystal structures [6,7], and most likely predominates in solution. Thus, the population of RS* is small, implying that the apparent rate of cleavage includes both the conformational change and the chemical step. The 2′ modifications described here presumably inhibit cleavage by shifting K_{eq} further to the left.

The 2′ modification data can give some information about the structure of RS* and the nature of the conformational change. When the six 2′ positions sensitive to bulk modification are mapped on the crystal structure [7] (Figure 4), they appear on two patches on opposite sides of the hammerhead. The three 2′ positions of residues $U_{1.1}$, C_3, and U_4 (shown in red) are on the minor groove side of domain I and near the cleavage site phosphate 1.1 (shown in green). The three 2′ positions of G_{12}, A_{14}, and $A_{15.1}$ (in red) are on the widened minor groove side of domain II, across the domain from the essential phosphate 9 (in green). However, it is striking that many of the nearby 2′ positions in the minor grooves of stem I and stem II (shown in blue) can accommodate bulky substituents without affecting cleavage. It is thus clear from Figure 4 that rearrangement of the crystal structure to form RS* will involve bringing parts of both core domains close together in a new way without involving the minor grooves of stems I and II. Although it would seem convenient to suggest rearrangement involving docking of the minor groove faces of both core domains so that the six essential residues are proximal to one another, this would involve a very large disruption of the hammerhead core, likely preventing the close approach of P1.1 and P9 suggested by Wang [13]. Alternate models for RS* could involve docking of the minor groove side of one of the domains with the major groove side of the

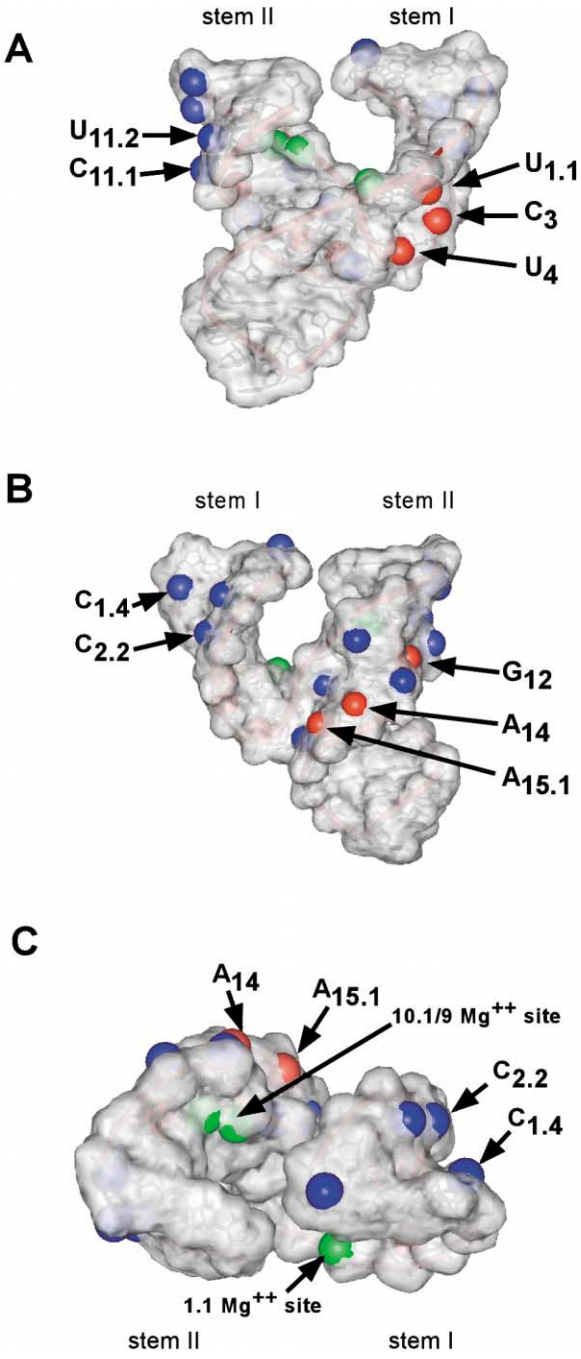

Figure 4. Three Views of the Context of 2′-Modifications Tested within the Hammerhead Crystal Structure [7]

Residues where a large 2′-modification inhibits cleavage are shown in red; residues where bulky 2′-modifications have no effect are shown in dark blue.

(A) View from the minor groove side of domain I.

(B) View from the minor groove side of domain II, after 180° rotation around the longitudinal axis.

(C) View from the "top" of stems I and II. The two essential metal-ion binding sites, 10.1/9 and 1.1, are shown in green in all three views.

other and a simultaneous more local conformational change in the other minor groove of the core. Although several models for RS* remain possible, they must satisfy the biochemical data presented. The development of methods to stabilize RS* for structure determination are clearly of high priority.

Significance

The hammerhead ribozyme is a small RNA catalyst that can be designed to cleave a specific RNA sequence in vitro or in vivo [1,2]. The mechanism by which the hammerhead catalyzes this reaction is largely unclear. Numerous inconsistencies between the available crystal structures [6,7] and the biochemical data identifying essential functional groups suggest that a significant conformational change must occur en route to the transition state [8]. Understanding this conformational change will lead to a more thorough understanding of the hammerhead catalytic mechanism.

A steric interference modification approach is herein used to reveal regions of the hammerhead that are involved in conformational change during the reaction. Bulky modifications were incorporated at unique 2′ positions in the hammerhead which were known to tolerate a 2′-deoxy modification without affecting cleavage. Several of these 2′ modifications significantly decrease the rate of cleavage, even though they can be fit into the crystal structure without causing steric clash. We conclude that two of the regions where such 2′-bulk effects are observed are involved in the formation of an alternate structure during the approach to the transition state where the extra bulk cannot be tolerated. These results confirm that during catalysis, both domains I and II within the hammerhead catalytic core are involved in a conformational change.

This steric interference modification approach can be used with other RNA molecules where the details of folding or conformational changes are not well understood. For an RNA where the structure and the 2′-hydroxyl requirements are known, this approach could reveal important conformational changes or help to distinguish between alternate folded states. Further, this steric interference approach could potentially be extended to probe the interface of RNA-protein or RNA-small molecule interactions.

Experimental Procedures

Materials

2′-amino-cytidine phosphoramidite was a generous gift from Wolfgang Pieken (Proligo). Acetic, propionic, and butyric acids were purchased as reagent grade from Sigma Chemicals. The synthesis of 3-(Phenyldithio)-propionic acid is described elsewhere [22]. Ethyl-isocyanate was purchased from Aldrich Fine Chemicals. 1-Ethyl-3-[3-dimethylaminopropyl]-carbodiimide was purchased from Pierce Biochemicals. Dithiothreitol was purchased from Fisher Biochemicals.

RNA Synthesis and Purification

The 2′-amino cytidine-containing oligonucleotides were synthesized on an Applied Biosystems Incorporated 394 oligonucleotide synthesizer using standard phosphoramidite chemistry and deprotected as previously described [39]. 2′-amino uridine-containing oligonucleotides were purchased from Dharmacon research and deprotected as previously described [40]. All deprotected 2′-amino-pyrimidine containing RNAs were purified by fractionation on 20% denaturing PAGE. After elution overnight from the gel in a buffer containing 50 mM bis-Tris (pH 6.5), 1 M NaCl, and 10 mM EDTA, the RNAs were ethanol precipitated and resuspended in water.

2′-O-allyl-containing RNAs were provided as a generous gift from Ribozyme Pharmaceuticals. These oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis as described above. The masses of all modified hammerheads were confirmed using MALDI-TOF mass spectrometry, with a 2′-hydroxy-piccolinic acid matrix. All spectra showed only one peak of appropriate mass.

Amidation of 2′-Amines

For amidation, all 2′-amino-containing RNAs were incubated for 1 hr at 37°C in 200 μL of a solution containing 100 mM MES (pH 6.5), 20 μM RNA, 20 mM carboxylic acid, and a 1:10 dilution of a freshly prepared aqueous solution of 150 mM EDC. After ethanol precipitating and resuspending the RNA, the amidation reaction was repeated, followed by ethanol precipitating and resuspending in a volume to give an approximate concentration of 50 μM. The concentration of the coupled product was determined by UV absorbance, and the identity of the product was verified by MALDI-TOF MS, using 2′-hydroxy-piccolinic acid matrix. All spectra showed only one peak, corresponding to the correct product.

Coupling of Ethyl-Isocyanates to 2′-Amines

Ethyl-isocyanate was coupled to 2′-amino-containing RNA as previously described [24]. A 50 μM solution of 2′-amino-RNA in pH 8.6 borate buffer was combined with an equal volume of 60 mM ethyl-isocyanate in dimethylformamide, incubated for 4 hr on ice, and ethanol precipitated. After resuspending the RNA, the identity of 2′-ethyl-ureido RNA was verified by MALDI-TOF MS, using 2′-hydroxy-piccolinic acid matrix. All spectra showed only one peak, corresponding to the correct coupled product.

Single-Turnover Kinetic Measurements

Single-turnover kinetic measurements of 2′-modified RNAs were performed as previously described [23]. 2.5 μM of ribozyme strand was preequilibrated with trace (<20 pM) 5′-[^{32}P]-labeled substrate strand in 100 mM HEPES (pH 7.5), 1 mM EDTA at 95°C for 5 min, followed by a slow-cooling to 37°C over 1 hr. After equilibrating at room temperature for 10 min, cleavage was initiated by adding either $MgCl_2$ to a final concentration of 10.93 mM or LiCl to a final concentration of 2 M or 4 M. Reactions were quenched by diluting into 5 volumes of a quench containing 50 mM EDTA in 80% formamide on ice and fractionated on 20% denaturing PAGE. Bands were quantitated using a Molecular Dynamics Storm phosphorimager. The fraction cleaved was plotted as a function of time and fit to the following equation:

$$(P/(P + S))_t = (P/(P + S))_0 + (P/(P + S))_\infty[1 - \exp(-k_2 t)]$$

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