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Functional groups on the cleavage site pyrimidine nucleotide are required for stabilization of the hammerhead transition state

NARAYAN BAIDYA,[1] GLENYSS E. AMMONS,[1] JASENKA MATULIC-ADAMIC,[2] ALEXANDER M. KARPEISKY,[2] LEONID BEIGELMAN,[2] and OLKE C. UHLENBECK[1]

[1]Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215, USA
[2]Ribozyme Pharmaceuticals, Inc., 2950 Wilderness Place, Boulder, Colorado 80301, USA

ABSTRACT

The role of individual functional groups on cytidine 17 in the hammerhead ribozyme was assessed by introducing modified pyrimidines into two kinetically well-characterized hammerheads. As long as the pyrimide ring size was maintained, the modifications had no effect on substrate binding, suggesting that the C17–C3 hydrogen bond observed in the X-ray structure is energetically neutral. However, modification of the exocyclic amino group and the carbonyl of C17 reduced the cleavage rate significantly, indicating that these groups are important in stabilizing the transition-state structure. C17 modifications did not affect the ratio of the forward and reverse reaction rates. Thus, unlike that believed previously, C17 is another one of many hammerhead residues critical in maintaining its active structure.

Keywords: catalytic RNA; ribozyme; structure–function

INTRODUCTION

RNA cleavage by the hammerhead ribozyme involves attack of a certain 2′ oxygen on the adjacent phosphate to give 2′, 3′ cyclic phosphate and 5′ hydroxyl termini (Buzayan et al., 1986; Hutchins et al., 1986; Prody et al., 1986; Uhlenbeck, 1987). Because cleavage is accompanied by inversion of stereochemical configuration about the phosphorous (van Tol et al., 1990; Slim & Gait, 1991), the 2′ oxygen is believed to attack the phosphorous in line with the leaving 5′ oxygen. However, the crystal structures of two quite different hammerhead sequences revealed identical cleavage site structures where the positions of the 5′ oxygen, phosphate and 2′ oxygen were not close to an in-line configuration (Pley et al., 1994; Scott et al., 1995). It does not appear that the 2′ H or 2′-O-methyl groups present in these hammerheads are responsible for the "incorrect" configuration because a recent crystal structure of an all RNA hammerhead obtained in the absence of divalent ions was quite similar (Scott et al., 1996). Finally, preliminary NMR data indicates that the solution structure of the hammerhead in the neighborhood of the cleavage site is not substantially different from the crystal structures (J.P. Simorre, N. Baidya, O.C. Uhlenbeck, & A. Pardi, in prep.). Thus, it is generally believed that the hammerhead structure must undergo at least a local rearrangement to achieve the active in-line configuration.

In considering the types of structural changes that might occur, it is striking that the cleavage site nucleotide C17 is stacked upon the inner base pair of helix I and makes a single hydrogen bond from its exocyclic amino group to the N_3 of the essential residue C3 in the catalytic core (Fig. 1). These interactions appear to hold the scissile ribose–phosphate linkage in the configuration incompatible with in-line attack. Because the scissile bond is on the outside of the structure and unavailable for interactions with the rest of the molecule, most models for hammerhead cleavage involve disruption of the C17–C3 interaction in order to achieve the in-line configuration and place the scissile bond near other functional groups that would promote cleavage (Pley et al., 1994; Scott et al., 1995; Setlik et al., 1995).

In a recent extensive kinetic and thermodynamic analysis of hammerheads with the four natural nucleotides at the cleavage site (Baidya & Uhlenbeck, 1997), we noted that the comparison of the C17 hammerhead with the C17U mutation was particularly interesting.

Reprint requests to Olke C. Uhlenbeck, Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215, USA.

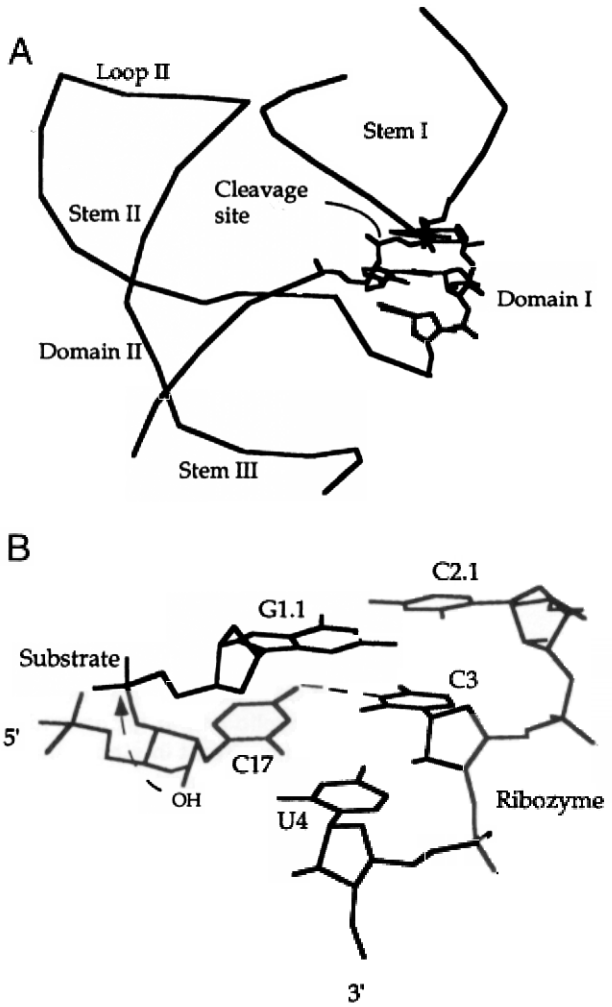

FIGURE 1. Structure of the hammerhead ribozyme (Pley et al., 1994). **A:** Overall arrangement of the RNA chain with the five residues near the cleavage site shown in detail. **B:** Enlargement of region near the cleavage site. Dotted arrow indicates the attack of the 2' hydroxyl on the scissile phosphate. Dotted line indicates the hydrogen bond between C17 and C3.

Despite the fact that the U17 hammerhead could not make an isosteric hydrogen bond with C3, substrate binding affinity was not changed, suggesting either that an alternative structure was formed or that the C3–C17 interaction was not thermodynamically significant. In addition, because the internal equilibrium of the U17 mutant was not changed, it appears that the structure of the cleaved U17 hammerhead is very similar to that of the cleaved C17 hammerhead. However, quite unexpectedly, the U17 hammerhead shows a 5- to 12-fold slower cleavage rate under a variety of solution conditions. This suggested that the exocyclic amino group and/or the N_3 of C17 participates in the stabilization of the conformation active in cleavage. In order to further explore this observation, we examined the cleavage properties of position 17 with the ultimate goal of identifying all the pyrimidine functional groups needed for stabilizing the transition-state structure.

RESULTS AND DISCUSSION

The nine modified pyrimidines, C-phenyl ribofuranoside, and the abasic ribonucleotide (Fig. 2) were introduced into position 17 of two kinetically well-characterized hammerheads, termed HH8 and HH16, that have identical catalytic cores, but differ in the length and sequences of stems I and III (Fig. 3). This approach permits the effect of the modifications on each of the rate constants that describe the hammerhead cleavage reaction to be determined (Baidya & Uhlenbeck, 1997). If only a single turnover of cleavage is considered, the bimolecular reaction consists of two reversible steps, the binding of the substrate oligonucleotide (S) to the ribozyme (E) and the cleavage of substrate to the 5′ product (P1) and 3′ product (P2):

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E{\cdot}S \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} E{\cdot}P1{\cdot}P2.$$

The short arms of HH8 result in very rapid dissociation of substrate so that $k_{-1} \gg k_2$ and cleavage can be described by Michaelis-Menton kinetics where $k_{cat} = k_2$ and $K_M = k_{-1}/k_1$ (Fedor & Uhlenbeck, 1992). The long arms of HH16 result in the products remaining stably bound after cleavage and permitting determination of k_{-2} and $K_{eq}^{int} = k_2/k_{-2}$ (Hertel et al., 1994). The critical cleavage rate constant k_2 can be obtained with both hammerheads.

The cleavage rates of the 11 modified substrates of HH8 were determined using a trace concentration of labeled substrate and at least 0.5 μM ribozyme, which is saturating for the C17 substrate. Although the cleavage rates varied dramatically, all substrates except the abasic nucleotide ultimately cleaved to greater than 90% with a single rate constant that is expected to equal k_2, the rate of chemical cleavage (Table 1). To confirm that saturation was achieved, the rates were determined at a higher ribozyme concentration and were found to be the same. For substrates that cleaved reasonably rapidly ($k_2 > 0.1\ min^{-1}$), the cleavage rates were determined for a series of ribozyme concentrations and the data were analyzed by an Eadie-Hofstee plot to obtain K_M and confirm k_2 (Fig. 4A for 2-pyridinone). Because $K_M = K_d$ for HH8 (Fedor & Uhlenbeck, 1992), these K_M values provided the substrate binding affinities in Table 1.

For slowly cleaving substrates of HH8, it was not possible to obtain an accurate K_M because the cleavage rates at low ribozyme concentrations became extremely slow. Consequently, substrate binding affinities were either determined by competitive inhibition experiments or by native gel electrophoresis. The former method involves measuring the cleavage rate of a trace concentration of the C17 substrate by a subsaturating ribozyme concentration in the presence of varying concentrations of nonradioactive N17 substrate (Dahm &

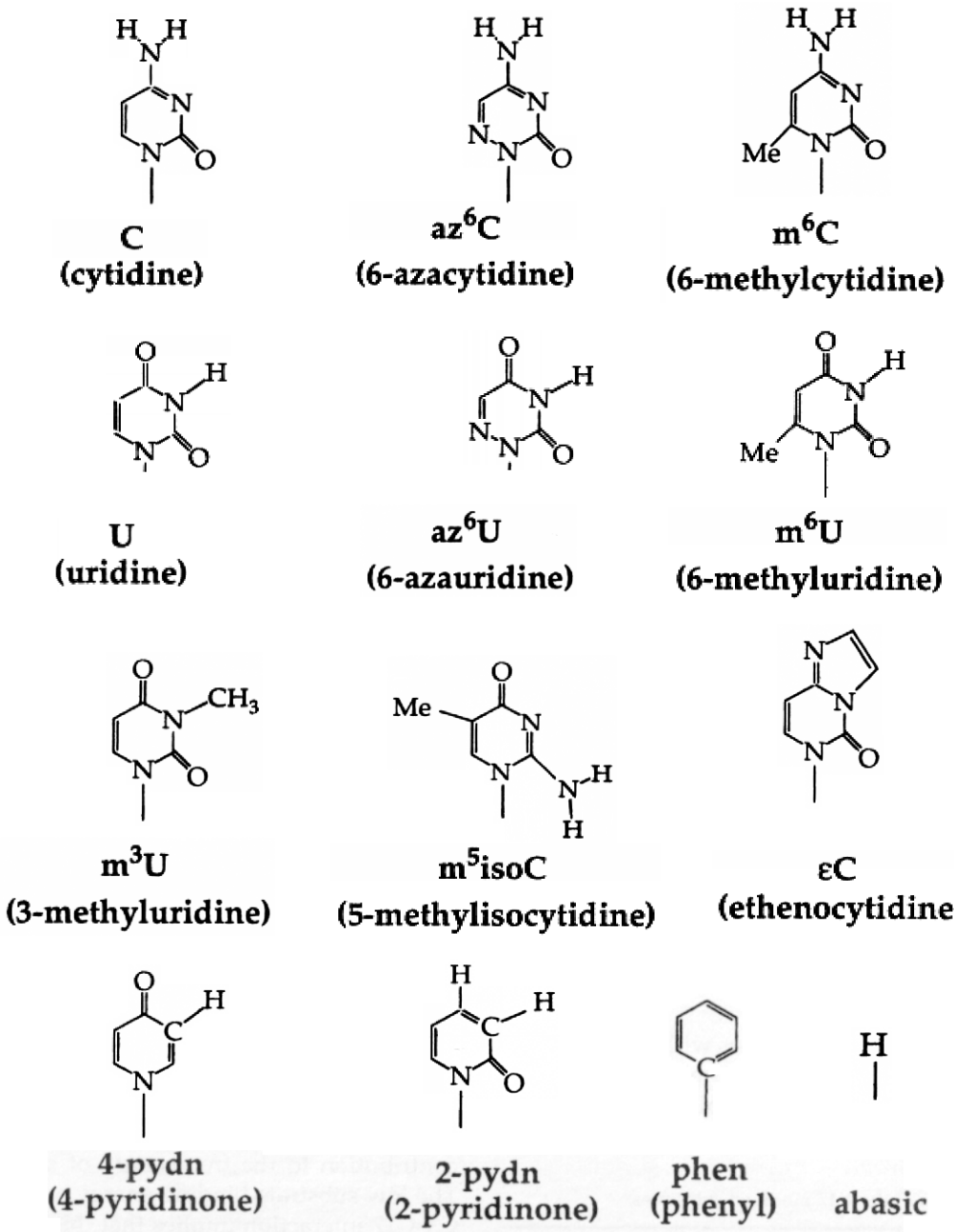

FIGURE 2. Structure of modified bases introduced at position 17 of hammerheads 8 and 16.

Uhlenbeck, 1990; Baidya & Uhlenbeck, 1997). By plotting the fraction of inhibition as a function of substrate concentration, a K_I is obtained (Fig. 4B for the 4-pyridinone substrate). As long as the modified competitor RNA does not cleave significantly during the course of the experiments, K_I is equal to the substrate binding affinity. The latter method involves incubating a trace concentration of labeled substrate with varying concentrations of ribozyme and analyzing the products on a nondenaturing gel (Pyle et al., 1990). By plotting the fraction of substrate bound versus ribozyme concentration, a K_d is obtained (Fig. 4C for the 4-pyridinone substrate). This method also requires that the modified substrate not cleave during the incubation and lengthy gel electrophoresis time. As a result, the K_d determination by nondenaturing gels is only appropriate for substrates that cleave at very slow rates. In several cases, K_I and K_d were determined for the same substrate with satisfactory agreement (Table 1).

All but one of the modified pyrimidines at the HH8 cleavage site show substrate binding affinities between 30 and 100 nM, which are within experimental error of one another and similar to the values obtained previously for C17 and U17. The only exception is the 10-fold weaker binding of the ethenocytidine derivative, which is similar to the K_d observed for the A17

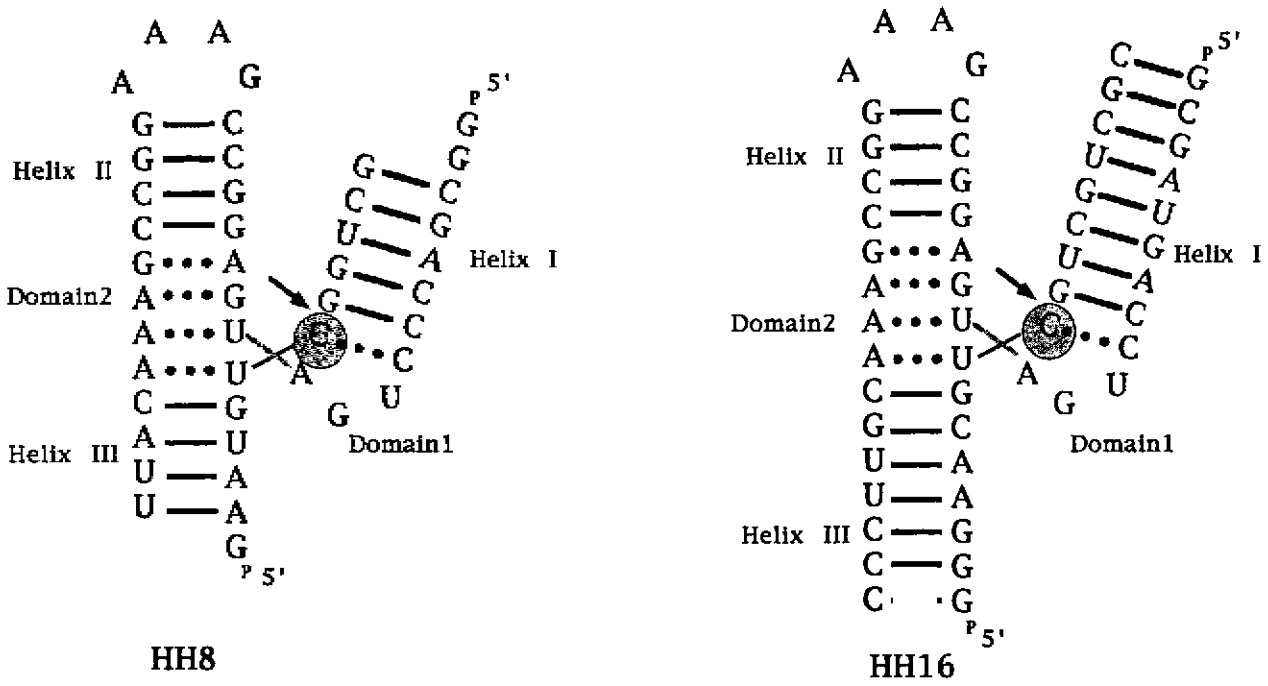

FIGURE 3. Secondary structures of hammerhead 8 (HH8) and hammerhead 16 (HH16) in a format that reflects the crystal structure. The substrate oligonucleotide is indicated in darker letters and the arrow denotes the cleavage site. Shaded circle marks nt 17 5' to the cleavage site, which is cytidine in the reference hammerhead. Pairing of C17 with C3 is indicated by the dotted line.

substrate. Because NMR data suggest that the A17 hammerhead has a slightly different structure (J.P. Simorre, N. Baidya, O.C. Uhlenbeck, & A. Pardi, in prep.), it is reasonable to propose that the ethenocytidine hammerhead also rearranges its structure to accommodate the steric bulk introduced by the additional five-membered ring.

It is quite striking that substrates with a diverse collection of pyrimidines at position 17 all bind with similar affinity. Because the X-ray crystal structures have a well-defined hydrogen bond between C3 and C17, we suggested previously (Baidya & Uhlenbeck, 1997) either that this hydrogen bond did not contribute to substrate binding affinity, or that the U17 substrate formed an alternate U-C pair. Although similar alternate structures can be proposed for many of the pyrimidine −17 analogues, it seems less likely that they are all equivalent energetically. Furthermore, the phenyl-17 substrate cannot form any hydrogen bonds to C3. Thus, the simplest conclusion is that when N17 is a pyrimidine, the residue does not make a favorable contribution to the free energy of substrate binding. The low substrate binding energy associated with the C3–C17 interaction implies that this part of the structure could be rearranged without a large energetic cost.

TABLE 1. Properties of the N17 modified substrates.

N17 Cleavage site	Binding affinity (nM) HH8	k_2 (min^{-1}) HH8	k_2 (min^{-1}) HH16	K_{eq}^{int} HH16
C	50[a]	1.4	0.9	100
U	57[a]	0.3	0.07	120
εC	370[a]	0.4	0.25	130
2-pydn	60[a]	0.25	—	—
m^3C	70[a]	0.6	—	—
4-pydn	75[b], 90[c]	0.003	—	—
m^5isoC	40[b], 45[c]	1×10^{-4}	—	—
m^6C	43[a], 46[b]	0.06	0.04	580
m^6U	48[c]	0.001	0.0008	570
az^6C	50[a]	0.7	0.5	250
az^6U	55[c]	0.025	0.02	400
phen	44[b], 50[c]	0.07	0.05	350
abasic	30[b], 40[c]	2×10^{-5}	1×10^{-5}	580

[a] = K_M.
[b] = K_d.
[c] = K_I.

In contrast to the substrate binding data, several of the substituted pyrimidines show very large differences in their value of k_2. In contrast to the small difference in k_2 between U and C, m^6U and m^5isoC have k_2 values about 10^3-fold slower than their unmodified counterparts, emphasizing the importance of using modified nucleotides in structure–function experiments. A comparison of the k_2 values of the first five modified pyrimidines in Table 1 permits a reasonably clear definition of the functional groups on C17 that are required for rapid cleavage. As suggested by the difference between C and U, the exocyclic amino group of C increases k_2 about fivefold. 2-Pyridinone, which lacks this group, has a cleavage rate similar to U. The proton on the N_3 of U probably does not contribute to

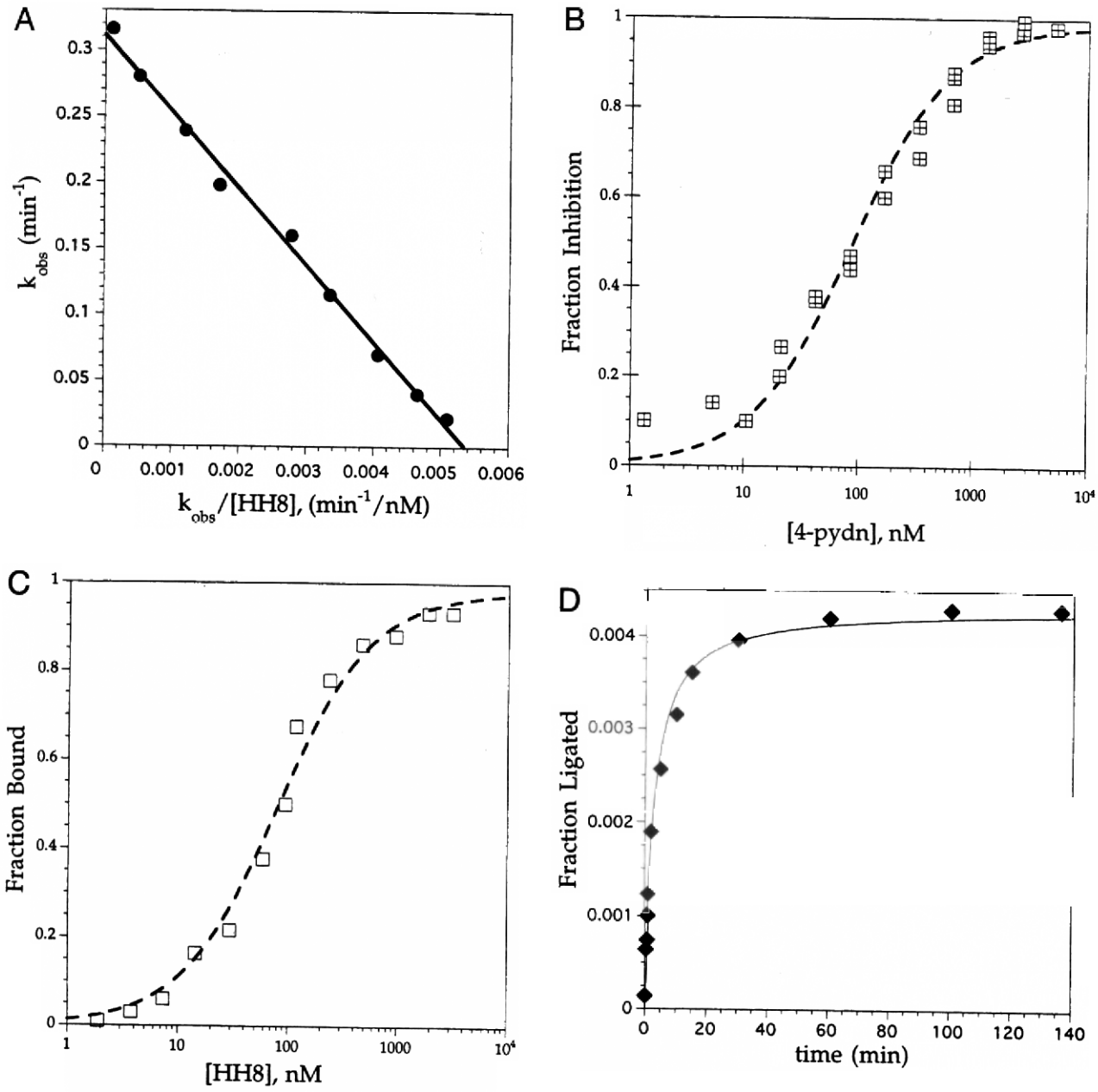

FIGURE 4. Determining rate and equilibrium constants for substrate analogues. All experiments were performed at 25 °C in 50 mM Tris HCl, pH 7.5, and 10 mM $MgCl_2$. **A:** Eadie–Hofstee plot of cleavage of a trace concentration of [5′-P^{32}] 2-pyridinone substrate of HH8 and varying concentrations (5–2,000 nM) of ribozyme. The line fits the data with k_{cat} = 0.31 min^{-1} and K_M = 60 nM. **B:** Competitive inhibition of cleavage of ^{32}P-labeled C17 substrate of HH8 and 10 nM ribozyme with varying concentrations of 4-pyridinone substrate. The rate of cleavage at each inhibitor concentration (k_{obs}) is used to calculate the fraction of inhibition ($1 - k_{obs}/k_C$), where k_C is the rate without inhibitor. The dotted line depicts a calculated competitive inhibition curve with K_I = 90 nM. **C:** Binding of trace concentration of ^{32}P-labeled 4-pyridinone substrate of HH8 with varying concentrations of ribozyme analyzed by native gel electrophoresis. Dotted line is a theoretical binding curve with K_d = 75 nM. **D:** Approach to the internal equilibrium in HH16 with 1 μM ribozyme, 2 μM P2, and a low concentration of [5′-P^{32}] P1 containing az^{6}C. The line fits a $k_{obs} = k_2 + k_{-2}$ = 0.3 min^{-1} and a fraction ligated ($1/K_{eq}^{int}$) of 0.0042.

k_2 because m^{3}U cleaves just as fast as uridine. The 2-carbonyl of C and U makes the largest contribution to k_2. This is best seen with 4-pyridinone, which is missing the 2-carbonyl and cleaves 200-fold slower than U. If the 2-carbonyl group is replaced by an amino group in m^{5}isoC, k_2 is even less, suggesting that an inhibitory interaction is introduced at this position.

The four different pyrimidines substituted at the 6 position were investigated for somewhat different reasons. At certain glycosidic angles, the methyl group of 6-methyl pyrimidines will clash with the ribose ring, thereby preventing certain conformations by hindering free rotation about the glycosidic bond (Suck & Saenger, 1972; Sundaralingam, 1975; Saenger, 1994).

Thus, m^6U and m^6C prefer the syn conformation around glycosidic bond. This effect would not occur for substitution of carbon with nitrogen, so the 6-aza pyrimidines closely resemble their natural counterpart and prefer the anti-conformation around glycosidic bond. Because the m^6C and m^6U hammerheads cleave 22-fold and 300-fold more slowly than their unmodified counterparts, it appears that the proper transition-state structure cannot be reached as easily with these hindered analogues. The az^6C control analogue cleaves virtually normally, suggesting that a productive interaction at C_6 of the pyrimidine ring is unlikely. The 10-fold slower cleavage of az^6U is probably due to the fact that, with a $pK_a = 6.9$ (Jonas & Gut, 1962), it is significantly deprotonated at pH 7.5. The resulting delocalized negative charge on the pyrimidine ring would lead to a less effective interaction with the critical carbonyl oxygen.

Considering the fact that the phenyl 17 substrate is missing both the 4 amino and 2-carbonyl groups, its cleavage rate is much faster than would be expected initially. Its $k_2 = 0.07\ min^{-1}$ is 23-fold faster than 4-pyridinone, which also lacks the two critical functional groups. This comparatively rapid rate of cleavage is unlikely to be a consequence of the C-C glycosidic linkage slightly altering the ribose conformation or the pK_a of the attacking 2′ hydroxyl because the uncatalyzed rate of cleavage is not significantly faster (data not shown). The high hydrophobicity of the C-phenyl riboside could also contribute to the comparatively rapid cleavage by altering the hydration around the adjacent scissile phosphodiester bond to create a lower dielectric environment (Egli et al., 1996; Narlikar & Herschlag, 1997).

Finally, the introduction of an abasic nucleotide at position 17 virtually abolishes specific cleavage. Based on experiments incubating the substrate oligonucleotide at pH 9, the uncatalyzed rate of cleavage at the abasic residue is not significantly slower than at other positions in the molecule (data not shown). Thus, the absence of a base appears not to affect the pK_a of the attacking 2′ hydroxyl group significantly. Because the abasic hammerhead substrate cleaves at least 150-fold slower than the 4-pyridinone substrate, it appears that, in addition to the critical functional groups, the transition state is also stabilized by stacking of the N17 ring. Attempts to restore cleavage by the addition of high concentrations of various purine or pyrimidine bases (Peracchi et al., 1996) were not successful.

Many of the modifications were also introduced into the substrate of HH16. The corresponding k_2 values at saturating (1 μM) ribozyme concentration were generally in good agreement with the HH8 values, considering that the basal rate of HH16 is 2- to 4-fold slower (Table 1). For each substrate, the corresponding ^{32}P-labeled P1 oligomer was purified from a preparative reaction and then recombined with saturating ribozyme and P2 to measure the extent of ligation (Fig. 4D for az^6C) and thus calculate K_{eq}^{int} (Hertel et al., 1994). In the case of substrates that cleaved very slowly ($k_2 < 10^{-3}\ min^{-1}$), the values of K_{eq}^{int} are likely to be overestimates because, during the long incubation time required to reach equilibrium, a fraction of the 2′, 3′ cyclic phosphate on the terminus of P1 will hydrolyze (Hertel & Uhlenbeck, 1995), making it unavailable for reversal. The values of K_{eq}^{int} are given in Table 1.

Most of the N17 modifications showed K_{eq}^{int} values that were only slightly greater than for C or U. This is not surprising because even the A17 hammerhead, which shows weaker substrate binding, has a similar value of K_{eq}^{int}. The relative free energy of the cleaved and uncleaved hammerheads appears not to be affected by residue 17. The equilibrium is in the direction of cleavage because a large favorable entropy change overcomes the unfavorable enthalpy associated with forming the strained cyclic phosphate product (Hertel & Uhlenbeck, 1995). The source of the entropy change is partly due to the weaker binding of a divalent ion to the cleaved hammerhead (Long et al., 1995), but primarily due to other unknown factors, such as increased molecular motions after cleavage. The contribution of residue 17 to these effects appears to be minimal.

Our experiments with modified pyrimidines have revealed that both the carbonyl and amino groups of cytidine 17 are used to stabilize the transition state of hammerhead cleavage, but make little or no contribution to substrate or product binding. Interactions that stabilize in the transition state with respect to the ground state are essential for catalysis. Because the cytidine ring does not participate directly in hammerhead cleavage chemistry, the carbonyl and amino functional groups must help to position the adjacent scissile ribose–phosphate bond correctly with respect to the remainder of the hammerhead core by making interactions that only occur in the transition state.

What kind of interactions could the functional groups of the cytidine be making? The most likely possibility is that they form hydrogen bonds with other functional groups in the hammerhead. Our substitution data suggest that the amino group and carbonyl group contribute about 1.0 and 2.5 kcal/mol, respectively, to transition-state stabilization, well within the range of hydrogen bonding energies. Another possibility is that C17 could be binding a divalent metal ion. There is precedent for pyrimidine functional groups coordinating metal ions in tRNA, either directly, in the case of lead (Brown et al., 1985), or through water, in the case of magnesium (Holbrook et al., 1977; Hingerty et al., 1978). Scott et al. (1996) noted the proximity of the C_2 carbonyl of C17 to a bound magnesium in the hammerhead crystal structure and proposed that this magnesium was essential for catalysis. However, because k_2 is only reduced about 300-fold when the C_2 carbonyl

is removed, it is unlikely that such a metal ion participates directly in the cleavage mechanism. If an essential "catalytic" metal were no longer able to bind, a much larger reduction in k_2 would be expected. For example, when the pro-R phosphorothioate is introduced at the scissile phosphate, the cleavage rate is reduced at least 10^4-fold, presumably due to the inability of a catalytic metal to bind (Dahm & Uhlenbeck, 1990).

Although most models for hammerhead cleavage propose a change in the position of C17 in order to reach the transition state (Pley et al., 1994; Scott et al., 1995; Setlik et al., 1995), none explicitly use both the C17 functional groups we have identified. It is important to note that, although all of these models propose that the transition state is reached with a relatively modest rearrangement from the X-ray crystal structure, there is no reason to believe that this is the case. Indeed, there is a great deal of biochemical data reporting large reductions in the k_2 for hammerheads modified at sites quite far from the cleavage site (Burgin et al., 1996; McKay, 1996). For example, certain modifications of G5 (Tuschl et al., 1993) or phosphate 9 (A. Peracchi, L. Beigelman, E. Scott, O.C. Uhlenbeck, & D. Herschlag, in prep.) reduce k_2 by 10^3-fold or more. It, therefore, seems possible that a quite large rearrangement of the crystal structure may occur in order to reach the transition state. The functional groups of C17 and many other residues would cooperate to transiently stabilize the active structure. In this view, C17 would not have a special role in hammerhead cleavage, which is more consistent with the well-defined, but comparatively modest effect upon the cleavage rate when C17 is modified.

MATERIALS AND METHODS

The substrate strands of HH8 and HH16 (Fig. 3) were synthesized chemically using phosphoramidite chemistry (Wincott et al., 1995). The modified phosphoramidite for ethenocytidine was obtained commercially (ChemGenes Corp, Weltham, Massachusetts) and the phosphoramidite for 5-methylisocytidine (Strobel et al., 1994) was a gift from S. Strobel (Yale University). The synthesis of phosphoramidites for 6-methylcytidine, 6-methyl uridine, 6-azacytidine, 6-azauridine (Beigelman et al., 1995b), 3-methyluridine (Zemlicka, 1970), 4-pyridinone ribonucleoside, 2-pyridinone ribonucleoside (Matulic-Adamic et al., 1996b), C-phenyl ribofuranoside (Matulic-Adamic et al., 1996a), and abasic nucleotide (Beigelman et al., 1995a) have been reported. After deprotection, each substrate oligonucleotide was purified by electrophoresis on 20% polyacrylamide gels containing 7 M urea, 5' ^{32}P-labeled with polynucleotide kinase and [γ-^{32}P] ATP, and repurified on denaturing gels (Fedor & Uhlenbeck, 1992). Fidelity of the incorporation of modified nucleosides at N17 was confirmed by base composition analysis, as described previously (Burgin et al., 1996). Ribozymes for HH8 and HH16 were prepared by transcription and purified as described previously (Fedor & Uhlenbeck, 1992; Hertel et al., 1994).

The methods for determining k_2 and K_M using trace substrate and varying the ribozyme concentration (Fedor & Uhlenbeck, 1992), K_I by competition with the corresponding C17 substrate (Dahm & Uhlenbeck, 1990; Baidya & Uhlenbeck, 1997), K_d with native gels (Pyle et al., 1990), and K_{eq}^{int} by the reverse reaction (Hertel & Uhlenbeck, 1995), have been described in detail. Experiments were performed in 10 mM $MgCl_2$, 50 mM Tris, pH 7.5, at 25 °C unless indicated.

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