Accelerated Publications

Hammerhead Ribozymes with a Faster Cleavage Rate†

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ABSTRACT: A hammerhead ribozyme that was previously reported to have a rate of chemical cleavage 10-fold faster than that of conventional hammerheads was analyzed in greater detail. Although originally found as a bimolecular hammerhead assembled through helices I and II, fast cleavage was observed in hammerheads in the more conventional helix I−helix III form, provided the sequence of helix I of the fast hammerhead was preserved. Mutations indicated that the fast rate of cleavage was due to the presence of both the U1.1-A2.1 and A1.2-U2.2 base pairs. The faster rate of cleavage was due to a small increase in the activation entropy of the reaction. In addition, we confirmed previous reports that increasing the length of helix I by greater than five base pairs inhibits cleavage slightly and have uncovered a similar effect in helix II.

The hammerhead ribozyme is a small RNA motif composed of a conserved catalytic core of 13 nucleotides that forms at the junction of three RNA helices (Symons, 1992). Cleavage of the RNA chain at a unique location in the core has been reported in unimolecular (Hutchins et al., 1986; Forster et al., 1988), bimolecular (Uhlenbeck, 1987; Haseloff & Gerlach, 1988; Jeffries & Symons, 1989), and even trimolecular (Koizumi et al., 1988) reactions involving hammerheads with many different helix lengths and sequences. In those cases where the chemical cleavage step has clearly been identified, every hammerhead has a very similar cleavage rate. In the frequently used ("standard") reaction conditions of 10 mM $MgCl_2$, pH 7.5, and 25 °C, a cleavage rate between 0.5 and 2 min^{-1} has been reported for at least 20 different hammerheads (Fedor & Uhlenbeck, 1990, 1992; Perreault et al., 1990; Slim & Gait, 1991; Heidenreich & Eckstein, 1992; Hendry et al., 1992; Sawata et al., 1993; Shimayama et al., 1993; Hertel et al., 1994; Woisard et al., 1994; Chartrand et al., 1995; Hendry & McCall, 1995; Bassi et al., 1996; Jankowsky & Schenzer, 1996). Faster cleavage rates have been reported for four additional hammerheads (Sawata et al., 1993; Chartrand et al., 1995; Shimayama et al., 1995a), but the measurements were performed at higher pHs, temperatures, or magnesium concentrations where the chemical step is known to be faster. When these faster rates are corrected to standard conditions using available data on the pH, temperature, and magnesium dependence of the cleavage rate (Dahm & Uhlenbeck, 1991; Perreault et al., 1991; Dahm et al., 1993; Clouet-d'Orval et al., 1995; Hertel & Uhlenbeck, 1995), the cleavage rates for all four of these faster hammerheads also fall in the 0.5−2 min^{-1} range. While numerous hammerheads with much slower cleavage rates have also been reported (Haseloff & Gerlach, 1988; Fedor & Uhlenbeck, 1990, 1992; Koizumi & Ohtsuka, 1991; Goodchild, 1992; L'Huillier et al., 1992; Emerick & Woodson, 1993; Odai et al., 1994; Hendry & McCall, 1995), subsequent analyses of several of them have indicated either that the cleavage rate was not the chemical step (Dahm & Uhlenbeck, 1990; Fedor & Uhlenbeck, 1990) or that a portion of the ribozyme or substrate oligonucleotides were in an inactive conformation (Hendry & McCall, 1995). Thus, it appears that virtually all hammerheads have a similar cleavage rate, suggesting that the cleavage rate is specified by the conserved catalytic core and not by the helix sequence or the way the hammerhead is assembled.

In light of the above discussion, two recent reports of a hammerhead with an approximately 10-fold faster rate of chemical cleavage under standard conditions are especially intriguing. In one case, the faster rate was the result of replacing uridine 7 in the catalytic core with 4-pyridinone (Burgin et al., 1996). In the second case, a fast hammerhead termed HHα1 was discovered accidentally in a search for a bimolecular hammerhead that assembled through the formation of helix I and helix II (Clouet-d'Orval & Uhlenbeck, 1996). A closely related hammerhead (HHα5) did not show a faster rate of cleavage. Since the sequence of the catalytic core of HHα1 is identical to that of many other hammerheads, the increased rate of cleavage is probably due to its particular combination of helices and loops. The purpose of this paper is to identify the sequence elements in HHα1 responsible for the fast rate of cleavage and to attempt to gain insight into the reason for the surprising improvement in the catalytic rate. During this analysis, we uncovered an indication that increasing the length of helix II by greater than four base pairs reduces the rate of hammerhead cleavage.

MATERIALS AND METHODS

RNA Synthesis. The substrate RNA Sα1 and all ribozyme RNAs except Eα1 were synthesized by *in vitro* transcription of a synthetic DNA template with T7 RNA polymerase and subsequently gel purified (Uhlenbeck, 1987; Fedor & Uhlenbeck, 1992). The ribozyme RNA Eα1 and all the substrate RNAs, except Sα1, were chemically synthesized and gel

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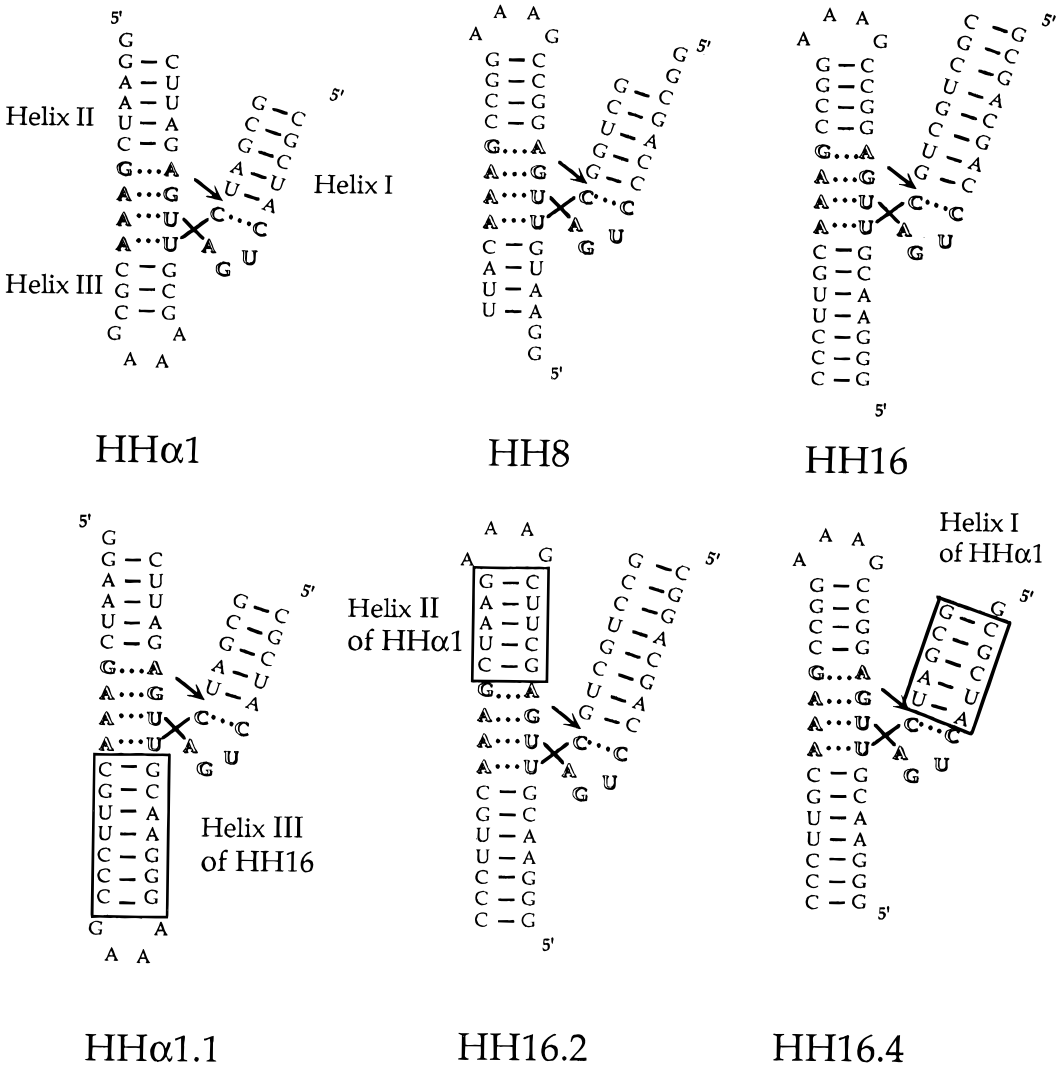

FIGURE 1: Sequences of the three "parent" hammerheads (above) and three hybrid stem hammerheads (below). Secondary structures are presented in a format that reflects the crystal structure (Pley et al., 1994; Scott et al., 1995). The open letters indicate the conserved catalytic core, and the arrow indicates the cleavage site. The dots indicate noncanonical pairs between nucleotides in the catalytic core.

purified (Fedor & Uhlenbeck, 1992); RNA substrates were 5′ end-labeled using T4 polynucleotide kinase and [γ-^{32}P]-ATP. For the RNA transcripts, the 5′ end terminal triphosphate was removed with alkaline phosphatase prior to labeling.

Kinetics. Single-turnover cleavage rates were measured with an excess of ribozyme and a trace amount of 5′-^{32}P end-labeled substrate. For a typical reaction, the appropriate concentration of ribozyme (0.5−2 μM) and <1 nM [5′-^{32}P]-substrate were heated at 95 °C for 2 min in 20 μL of 50 mM Mops (pH 7.5) or Pipes (pH 6.5) buffer and cooled to 25 °C in 5 min. For the zero time point, 1 μL of this mixture was removed and added to 10 μL of stop solution (8 M urea, 50 mM EDTA, 0.05% bromophenol blue, and xylene cyanol). The cleavage reaction was initiated by addition of 2 μL of 0.1 M $MgCl_2$ to give a final concentration of 10 mM. Aliquots (2 μL) were removed at specific time intervals and the reactions immediately quenched in 10 μL of stop solution. Substrate and products were separated on 20% polyacrylamide/7 M urea gels and quantitated by using a Molecular Dynamics phosphorimager. Cleavage rates were obtained as described previously (Fedor & Uhlenbeck, 1992) and varied by less than 20% in independent determinations. The cleavage rate of each ribozyme was confirmed to be independent of ribozyme concentration which demonstrates that saturation was achieved in single-turnover reactions.

RESULTS AND DISCUSSION

Figure 1 compares the sequences of the previously identified fast hammerhead, HHα1 (Clouet-d'Orval & Uhlenbeck, 1996), with two well-characterized conventional hammerheads, HH8 and HH16 (Fedor & Uhlenbeck, 1992; Hertel et al., 1994). Under standard reaction conditions (10 mM $MgCl_2$, pH 7.5, and 25 °C), the cleavage rates are 10 ± 2 min^{-1} for HHα1, 1.2 ± 0.2 min^{-1} for HH8, and 0.4 ± 0.1 min^{-1} for HH16 which agree well with previous determinations. Under these reaction conditions, the fast cleavage rate of HHα1 can only be measured accurately under multiple-turnover conditions. Under single-turnover conditions using trace concentrations of substrate and excess enzyme, HHα1 is too fast to measure by manual methods. However, the cleavage rates of many of the hammerheads in this study can only be determined under single-turnover conditions because product release is rate-limiting under multiple-turnover conditions. Thus, cleavage experiments were all carried out at pH 6.5 where hammerhead cleavage rates are about 10-fold slower. Under these conditions, the cleavage rates are 1.2 ± 0.3 min^{-1} for HHα1, 0.1 ± 0.02 min^{-1} for HH8, and 0.04 ± 0.01 min^{-1} for HH16 (Table 1).

Table 1: Hammerhead Cleavage Rates

hammerhead[a]	cleavage rate[b] (min^{-1})	rate relative to HHα1
HHα1	1.2 ± 0.3	(1)
HHα1.1	1.2 ± 0.2	1.0
HH16	0.04 ± 0.01	0.03
HH16.2	0.010 ± 0.005	0.008
HH16.4	1.1 ± 0.2	0.92
HH16.41	0.5 ± 0.1	0.42
HH16.42	0.7 ± 0.1	0.59
HH16.43	0.3 ± 0.1	0.25
HH16.44	0.6 ± 0.1	0.5
HH16.46	1.1 ± 0.2	0.92
HH16.47	0.8 ± 0.06	0.68
HH8	0.1 ± 0.02	0.08
HH8.1	0.9 ± 0.2	0.75

[a] Hammerhead sequences shown in Figures 1 and 2. [b] Cleavage rates determined in 10 mM $MgCl_2$ and 50 mM Pipes at pH 6.5 and 25 °C.

Defining the Critical Sequence Elements. In order to define the elements of HHα1 responsible for the fast rate of cleavage, three hybrid hammerheads were designed that exchange helices between HHα1 and HH16 (Figure 1). The rates of cleavage of these hybrid hammerheads (Table 1) clearly indicate that the fast rate of cleavage of HHα1 is entirely due to helix I. Thus, the introduction of the long helix III of HH16 into HHα1 to form HHα1.1 does not alter its fast cleavage rate. Similarly, the introduction of helix II of HHα1 into HH16 to form HH16.2 does not affect cleavage. However, the introduction of helix I of HHα1 into HH16 to form HH16.4 increases its rate of cleavage so it is just as fast as that of HHα1.

To identify which part of the sequence of helix I was responsible for the fast cleavage, mutations were made which altered individual base pairs in helix I of HH16.4 (Figure 2) and their cleavage rates determined (Table 1). From these experiments, it appears that the two innermost base pairs of helix I are primarily responsible for the fast cleavage rate. Thus, inversion of either the U1.1-A2.1 pair (HH16.43), the A1.2-U2.2 pair (HH16.41), or both (HH16.42) reduced the rate of cleavage significantly. Inverting the positions of the 1.2-2.2 and 1.3-2.3 pairs (HH16.44) also slows cleavage. In contrast, inverting outer pairs C1.4-G2.4 (HH16.46) and G1.5-C2.5 (HH16.47) maintains the fast cleavage rate.

In order to confirm that the U1.1-A2.1 and A1.2-U2.2 base pairs result in a faster cleavage rate, they were inserted into HH8 (Figure 2) to produce HH8.1. Due to the resulting low stability of helix I of HH8.1, much higher concentrations of ribozyme were needed to reach saturation. Nevertheless, its cleavage rate was nearly 9-fold faster than that of HH8, supporting the view that the two U-A pairs that were introduced into the two internal positions of helix I promote the more rapid cleavage of any hammerhead.

Although not every combination was tested, it is possible that the U1.1-A1.2 dinucleotide (with its corresponding pairing partners A2.1 and U2.2) is the combination that promotes the most rapid cleavage. In the data presented above, five of the 16 possible dinucleotides at positions 1.1 and 1.2 were tested and U-A showed the fastest cleavage rate. If one includes the many hammerheads that cleave between 0.5 and 2 min^{-1} discussed in the introductory section, seven additional dinucleotide sequences appear at these two positions and no "fast" cleavage rates are observed.

How could these two internal base pairs in helix I act together to modulate the cleavage rate in such a specific manner? While these two base pairs are not considered part of the catalytic core of the hammerhead, the 5′-oxygen of the ribose of residue 1.1 is the leaving group in the reaction mechanism. It is likely that the precise position of the leaving group is an important factor in the rate of the phosphoryl transfer reaction. Since different dinucleotide pairs in an RNA helix can have significantly different backbone structures (Bubienko et al., 1983), it is possible that the two U-A base pairs in the hammerhead have a structure that results in the 5′-oxygen being optimally placed in the transition state. Another factor that may contribute to the fast reaction rate is the fact that the U-A helical nearest neighbor interaction is quite weak (Freier et al., 1986). If partial disruption of one of the base pairs is needed to reach the transition state configuration, the U-A pairs may be able to do so more easily than other pairs. Thus, either the structural or dynamic properties of the U-A pairs may stimulate cleavage.

In order to further investigate the role of the U-A pairs on hammerhead cleavage, two additional experiments were performed. In the first, the temperature dependence of the cleavage rate was determined for a fast hammerhead containing the U-A pairs (HH16.4) and two slower hammerheads (HH16.41 and HH16.42) which contained mutations in these pairs. If the U-A pairs permit a more facile conformational change in reaching the transition state, an accompanying change in the activation energy may occur. Interestingly, this is not what is observed. Instead, between 2 and 37 °C, all three hammerheads gave linear van't Hoff plots with very similar activation energies (21 ± 1 kcal/mol) and calculated activation enthalpies (20 ± 1 kcal/mol). The values agree closely with those reported for HHα1 (Clouet-d'Orval & Uhlenbeck, 1996) and HH16 (Hertel & Uhlenbeck, 1995). Thus, the faster cleavage for HH16.4 primarily reflects a small (2−3 eu) but reproducable increase in the activation entropy. Such an increase in activation entropy could be due to many factors, including changes in the position or hydration of one of the metal ions that participates in the reaction or changes in the overall conformational flexibility. The absence of a change in activation enthalpy argues against structural changes that involve any large change in base stacking, which is generally accompanied by a significant enthalpy change (Freier et al., 1986).

A second experiment probing the role of the U-A pairs in hammerhead cleavage was examination of the effect of changing the identity of residue 17 in HH16.4. This experiment was motivated by both structural and kinetic considerations. In the X-ray crystal structures of the hammerhead, C17 forms a single hydrogen bond with C3 and both nucleotides are stacked upon the 1.1-2.1 base pair that is the focus of this work (Pley et al., 1994; Scott et al., 1995). However, recent NMR evidence suggests that a hammerhead with an A at position 17 has a somewhat different structure, where A17 is no longer close to the 1.1-2.1 pair, but is stacked on position 16.1 (Simorre et al., 1997). In addition, kinetic and thermodynamic data on HH8 and HH16 indicate that the reaction pathway is somewhat different when different nucleotides are present at position 17 (Baidya & Uhlenbeck, 1997). Table 2 compares the cleavage rates of HH16.4 containing U, C, or A at position 17 with the corresponding data with HH16 determined previously. G17 was not tested since it cleaves very poorly due to pairing with C3. Although there are some small differences between HH16 and HH16.4, there is no clear indication of any synergy between positons 17 and the

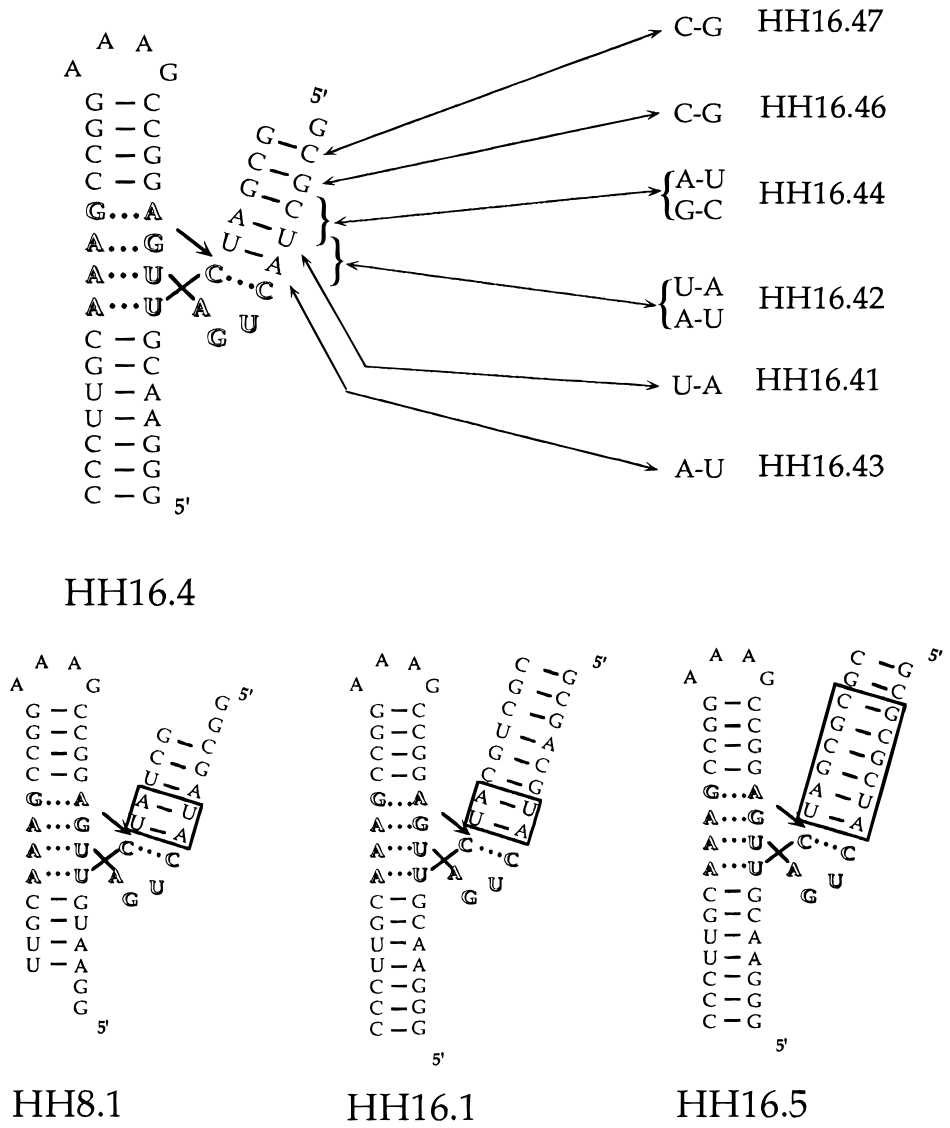

FIGURE 2: Hammerhead mutations in helix I in different backgrounds. The mutations are indicated by arrows (above) or enclosed in boxes (below).

Table 2: Cleavage Rates for N17 Mutations

hammerhead	C17	U17	A17
HH16.4[a]	1.1 ± 0.2	0.3 ± 0.02	2.1 ± 0.2
HH16[b]	0.9	0.07	0.4

[a] Cleavage rates determined in 10 mM $MgCl_2$ and 50 mM Pipes at pH 7.5 and 25 °C. [b] Data from Baidya and Uhlenbeck (1997) determined in 10 mM $MgCl_2$ and 50 mM Hepes at pH 7.5 and 25 °C.

adjacent U-A pairs in the transition state.

Helix Length Effects. The stimulation of cleavage by the U-A pairs is not as large when tested in the HH16 background. Thus, when either just the U-A pairs (HH16.1) or the entire helix I sequence of HHα1 (HH16.5) is transplanted into HH16 (Figure 2), the cleavage rate is only stimulated 2−3-fold compared to that of HH16 (Table 3), instead of the 25-fold observed with HH16.4 and 10-fold in the HH8 background (Table 1). Since HH16.4 is simply a truncated version of HH16.5, this reduced stimulation appears to be related to the length of helix I. When the ribozyme of HH16.5 was used with the substrate of HH16.4, the cleavage rate was relatively slow, but the ribozyme of HH16.4 could cleave the substrate of HH16.5 at the same rapid rate as it could its own shorter substrate (Table 3). This suggests that the length of the ribozyme strand of HH16.5 is responsible for the reduced rate.

Table 3: Helix I and Helix II Length and Sequence Effects

hammerhead	rate[a] (min^{-1})	rate relative to HH16.4
HH16	0.04 ± 0.01	0.04
HH16.4	1.1 ± 0.2	(1)
HH16.1	0.09 ± 0.01	0.08
HH16.5	0.10 ± 0.02	0.10
E16.4/S16.5	1.0 ± 0.1	0.9
E16.5/S16.4	0.25 ± 0.05	0.23
HH8	0.12 ± 0.02	0.11
HH8.2	0.032 ± 0.002	0.03
HH8.3	0.048 ± 0.003	0.04
HH16.48	0.17 ± 0.01	0.15
HH16.51	0.11 ± 0.03	0.1
HH16.52	0.16 ± 0.04	0.15
HH16.53	0.030 ± 0.005	0.027
HH16.54	0.012 ± 0.002	0.001

[a] Cleavage rates determined in 10 mM $MgCl_2$ and 50 mM Pipes at 6.5 and 25 °C.

The reduced stimulation by the introduction of U-A pairs in HH16 may be a manifestation of the small reduction in hammerhead cleavage rate when the length of helix I exceeds five or six base pairs. This phenomenon was reported by Hendry and McCall (1996) using three different hammerheads where the ribozyme oligonucleotides contained 10

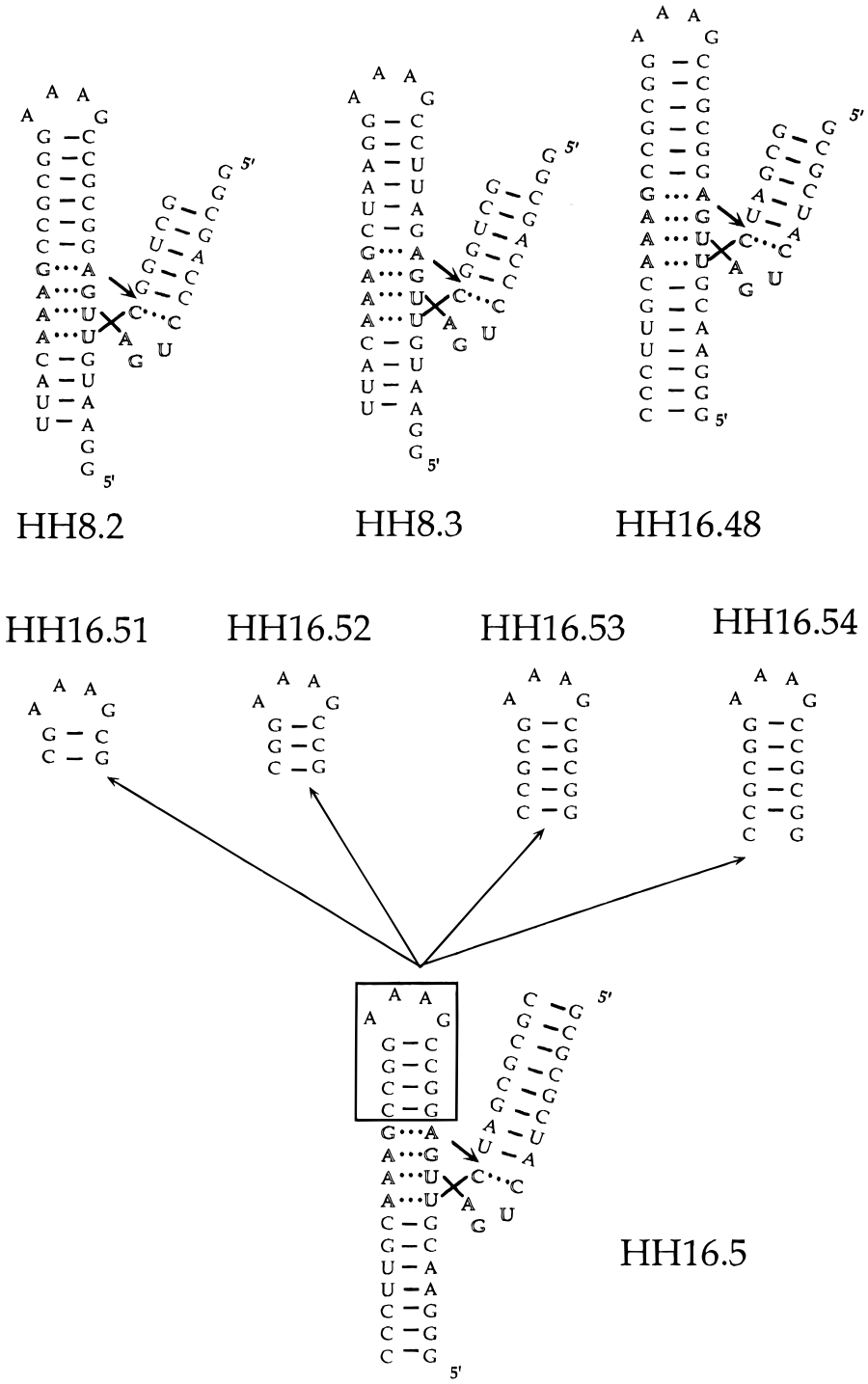

FIGURE 3: Hammerheads with helix II at various lengths. The mutations are indicated by the box.

nucleotides in helix I and helix III. Using a series of different substrate lengths, they found that the cleavage rate was reduced 3−15-fold when helix I exceeded six base pairs, whereas helix III showed no such length effect. While their results were with long ribozyme oligonucleotides, they are approximately consistent with our observation that HH16, which has eight base pairs in helix I, cleaves about 4-fold slower than HH8 which has five base pairs in helix I. In addition, a version of HHα1 that is elongated by two base pairs in both helix I and helix II also shows a 5-fold lower cleavage rate (T. Stage, unpublished experiments). It is not known what is responsible for this helix I length effect. The hammerhead crystal structures (Pley et al., 1994; Scott et al., 1995) reveal that the sugar−phosphate backbone of nucleotides 2.4 and 2.5 in helix I are close to the backbone of nucleotides 11.3 and 11.4 in helix II. This has led to the suggestion (Hendry & McCall, 1996) that, when helix I exceeds six base pairs, an interhelix interaction stabilizes an inactive hammerhead conformation and thereby reduces the apparent rate of cleavage. A similar inhibitory effect of peripheral sequences in hammerheads derived from natural RNAs has been reported (Miller et al., 1991; Hendry & McCall, 1995; Garrett et al., 1996). Perhaps such an inhibitory helix I−helix II interaction reduces the stimulatory effect of introducing the U-A pairs. When helix 1 is reduced to five base pairs or less, the inactive conformation cannot form and the full effect of the U-A pairs on increasing the cleavage rate is observed.

The possible interaction between helices I and II prompted an investigation of helix II length on hammerhead cleavage

activity. While several groups (Tuschl & Eckstein, 1993; Long & Uhlenbeck, 1994; Shimayama et al., 1995b) had shown that the length of helix II can be reduced without changing the cleavage rate, no experiments extending helix II have been reported. Thus, two types of experiments were performed. First, helix II of HH16.4 was extended from four base pairs to six base pairs (HH16.48), and helix II of HH8 was extended from four base pairs to six base pairs with two different sequences (HH8.2 and HH8.3) (Figure 3). For both these hammerheads, the longer helix II reduced the cleavage rate about 5-fold (Table 3).

The second experiment involved varying the length of helix II in HH16.5, which has a long helix I (Figure 3). In this case, when helix II is lengthened to either five (HH16.53) or six (HH16.54) base pairs, the cleavage rate is also reduced by about 5-fold (Table 3). In contrast, when helix II is reduced to three (HH16.52) or two (HH16.51) base pairs, no change in the cleavage rate is observed. This latter result confirms other reports holding that hammerheads with a stable, short helix II are fully active (Tuschl & Eckstein, 1993; Long & Uhlenbeck, 1994). Thus, in several different backgrounds, increasing the length of helix II beyond four pairs reduces the rate of cleavage and decreasing the length has no effect. Since the magnitude of the effect is similar to what is observed when helix I is lengthened, it is tempting to speculate that it, too, can be explained by the same inhibitory interhelical interaction. However, two results tend to argue against such a model. First, the helix II length effect is observed even when helix I is short where the interhelical contact is not thought to occur. Second, when helix I is long, reducing the length of helix II to less than four pairs might be expected to relieve the interhelical inhibition and result in faster cleavage, but this is not the case. In other words, the helix II length effect appears to be independent of the helix I length effect.

In summary, the 10-fold faster cleavage observed for HHα1 is due to the presence of U-A base pairs 3′ to the hammerhead cleavage site. The full effect of this sequence is only seen when both helix I and helix II are relatively short so that the inhibitory effect of long helices is avoided. Like the faster 4-pyridinone hammerhead studied previously (Burgin et al., 1996), we find that the improved cleavage rate required a precise modification at a nonessential site. Since the two inner base pairs of helix I are relatively far from postion 7 in the hammerhead crystal structure, it is possible that the two fast muations could act independently. If this is the case, a hammerhead containing both 4-pyridinone and the U-A base pairs would be expected to cleave at a rate approaching 100 min^{-1} under standard conditions. Such a hammerhead might show improved gene inactivation properties and would be an interesting candidate for structural studies.

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