A Kinetic and Thermodynamic Analysis of Cleavage Site Mutations in the Hammerhead Ribozyme†

Narayan Baidya and Olke C. Uhlenbeck*

Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215

Received August 26, 1996; Revised Manuscript Received November 22, 1996⊗

ABSTRACT: Two kinetically well-characterized hammerheads with different arm lengths were used to reinvestigate the cleavage properties of substrates with the four natural nucleotides at position 17, the residue 5′ to the cleavage site. From experiments measuring substrate binding affinity, cleavage rates. and the internal equilibrium, free energy profiles of the reaction of all four substrates were constructed. Each nucleotide at the cleavage site affects the energy profile quite differently. Whereas C and U have the same ground state energy, U destabilizes the transition state by 1 kcal/mol. A destabilizes both the ground and transition states by 1 kcal/mol, and G stabilizes the ground state by 2 kcal/mol and destabilizes the transition state by 4 kcal/mol. These data, along with experiments with the C3U mutant hammerhead, indicate that although an N3−N17 pair can form, the contribution to the binding energy for the wild-type (C3−C17) hammerhead is quite small. Thus, the energetic cost of disrupting the C3−C17 pair is not great, consistent with several proposals that this occurs during cleavage. The data also suggest that the structure in the transition state involves different stabilizing interactions with nucleotide 17 than those that are observed in the ground state. Finally, the A17 hammerhead may cleave by a slightly different reaction pathway.

The hammerhead ribozyme is a small RNA motif that catalyzes an intermolecular reaction in which a unique 2′-hydroxyl group in the RNA attacks the adjacent phosphate to generate 2′,3′-cyclic phosphate and 5′-hydroxyl cleavage products (Buzayan et al., 1986; Hutchins et al., 1986; Prody et al., 1986; Uhlenbeck, 1987). Like with many protein ribonucleases that catalyze the same reaction, hammerhead cleavage involves inversion of stereochemical configuration about the scissile phosphate (Perreault et al., 1990; van Tol et al., 1990; Slim & Gait, 1991), suggesting a mechanism where the 2′-oxygen attacks the phosphate in line with the leaving 5′-oxygen. Two recent X-ray crystal structures of the hammerhead ribozyme (Pley et al., 1994; Scott et al., 1995) show that the relative orientation of the ribose and phosphate at the cleavage site is incompatible with the in-line conformation needed for cleavage. Instead, the exocyclic amino group of the cytidine residue 5′ to the cleavage site (C17) forms a single hydrogen bond with the nitrogen-3 of cytidine 3 in the catalytic core, and the resulting C−C pair stacks on the end of helix 1 (Figure 1). As a result, the backbone conformation at the cleavage site resembles that in an A-type RNA helix, which is unfavorable for in-line attack (Figure 1B). Several of the models suggested for the cleavage mechanism (Pley et al., 1994; Scott et al., 1995; Setlik et al., 1995) invoke disruption of the C3−C17 pair and partial or complete unstacking of C17 from the adjacent G1.1−C2.1 base pair in order to obtain the in-line configuration.

The possible importance of the C17−C3 pair in the hammerhead cleavage mechanism has increased interest in the properties of hammerheads with mutations in these locations. Mutagenesis of C3 to any other nucleotide has been shown to result in a greatly reduced cleavage rate, identifying this residue as an important part of the hammerhead core (Koizumi et al., 1988b; Ruffner et al., 1990; Koizumi & Ohtsuka, 1991; Adams et al., 1994). Partly because of the potential application of the hammerhead in gene inactivation studies, mutagenesis of C17 has been examined by many groups (Koizumi et al., 1988a,b, 1989; Ruffner et al., 1990; Koizumi & Ohtsuka, 1991; Perriman et al., 1992; Nakamaye & Eckstein, 1994; Shimayama et al., 1995; Zoumadakis & Tabler, 1995). Although the results differed somewhat, there is general agreement that while C17, A17, and U17 are active, G17 is not. The poor cleavage activity of G17 has been hypothesized to be the result of a G17−C3 base pair (Ruffner et al., 1990; Koizumi & Ohtsuka, 1991; Perriman et al., 1992; Shimayama et al., 1995).

In this work, we have reinvestigated the effects of mutations in C17 and C3 on the hammerhead cleavage reaction. The primary motivation for doing this is that it is not possible to deduce mechanistic conclusions from the available activity data for two quite different reasons. First, many of the hammerheads previously studied showed very slow cleavage rates or incomplete cleavage, presumably due to the presence of alternative conformations of either the ribozyme or the substrate oligonucleotides. Second, the earlier experiments either obtained simple cleavage rates or, at best, determined k_{cat} and K_M. However, the only kinetic parameters that can be meaningfully related to the crystal structure are those describing elemental steps in the cleavage mechanism. The Michaelis−Menten parameters, k_{cat} and K_M, can each reflect different combinations of rate constants, depending upon the length of the hammerhead arms and initial reaction conditions chosen. The discovery of several

† This research was supported by NIH Grants GM 36944 and AI 30242 to O.C.U. and by Damon Runyan−Walter Winchell Postdoctoral Fellowship Grant DRG 1280 to N.B.

*Corresponding author.

⊗ Abstract published in *Advance ACS Abstracts,* January 15, 1997.

S0006-2960(96)02165-4 CCC: $14.00

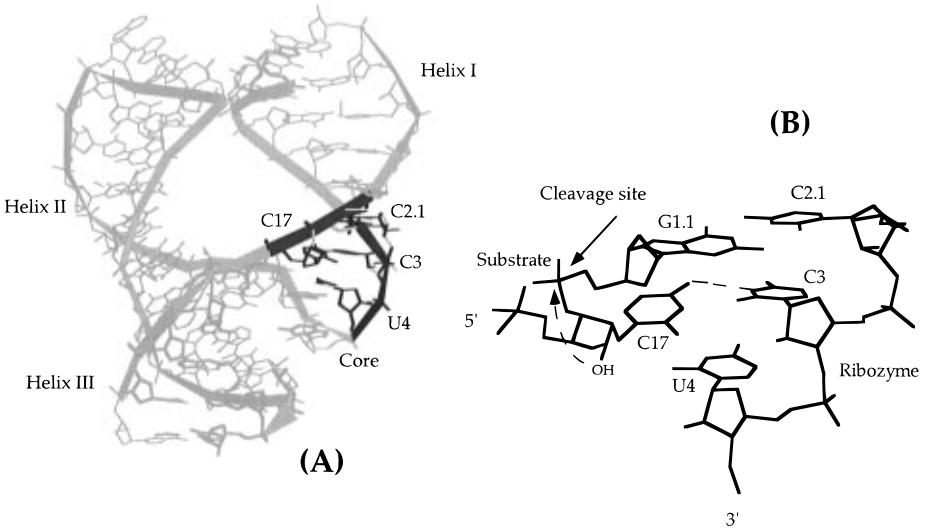

FIGURE 1: Structure of the hammerhead ribozyme near the cleavage site. (A) Overview of the hammerhead structure of Pley et al. (1994). The region around the cleavage site is darkened. (B) Detail of the cleavage site, showing the C17−C3 hydrogen bond and the relative positions of the attacking 2′−OH and the cleavage site phosphate (solid arrow). The dashed arrow indicates the reaction that must occur.

hammerheads that appear to be free of major alternative conformers and the development of methods to determine the elemental rate constants for the different steps in the cleavage reaction (Fedor & Uhlenbeck, 1992; Hertel et al., 1994) make it possible to meaningfully evaluate the effect of any hammerhead mutation on the cleavage pathway.

MATERIALS AND METHODS

Synthesis and Characterization of Oligonucleotides. The substrate RNAs were chemically synthesized using phosphoramidite techniques (Usman et al., 1987). Hammerhead ribozymes 8 and 16 were prepared by *in vitro* transcription from synthetic DNA templates using T7 RNA polymerase (Milligan et al., 1987). RNAs were purified by electrophoresis on 20% polyacrylamide gels containing 7 M urea, visualized by UV shadowing, cut out, eluted, and desalted using a C-18 column (Scaringe et al., 1990). Substrate RNAs were labeled at their 5′ termini using [γ-^{32}P]ATP and T4 polynucleotide kinase.

Cleavage Reactions. Single-turnover experiments with ribozyme in excess (550−2000 nM) and trace ^{32}P-labeled substrate were used to determine the rate constant for the cleavage reaction (Hertel et al., 1994). Most cleavage reactions were carried out in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 25 °C, as described previously (Fedor & Uhlenbeck, 1990). The ribozyme and substrate were first annealed together in Tris-HCl buffer by heating at 90 °C for 1.5 min and slowly cooling down to room temperature. Reactions were initiated by adding $MgCl_2$ to a final concentration of 10 mM. Aliquots (1 μL) were removed at appropriate intervals and quenched with 10 μL of denaturing quench buffer containing 90% (v/v) formamide, 50 mM EDTA, and 0.05% bromphenol blue and xylene cyanol dyes. Product separation, identification, and quantitation were done as described in Hertel et al. (1994). The experiments measuring the cleavage rate as a function of temperature were performed in 50 mM Hepes buffer, and the pH was adjusted to 7.5 at each temperature.

The inhibition of S8C cleavage by S8G was measured at concentrations of S8G from 0 to 500 nM, a trace concentration of labeled S8C, and subsaturating (10 nM) ribozyme. The fraction of inhibition at each S8G concentration was calculated as $1 - (k_{2S8G}/k_2)$, where k_{2S8G} and k_2 are the rate of cleavage in the presence and absence of S8G, respectively. The fraction inhibition versus concentration of S8G was plotted and fit to a bimolecular binding equation to obtain K_I.

The internal equilibrium constant K_{eq}^{int} was determined as described in Hertel and Uhlenbeck (1995) using 0.55 μM E16, 1.1 μM P2, and a trace concentration of 5′-^{32}P-labeled P1.

Measurement of Equilibrium Dissociation Constant for the E·S Complexes. The equilibrium dissociation constant for the complex between E8 and substrate S8G was measured using nondenaturing gel electrophoresis with some modification of the method of Pyle et al. (1990) described by Fedor and Uhlenbeck (1992). Trace amounts (picomolar) of 5′-^{32}P-labeled substrate were mixed with a series of 15 ribozyme concentrations ranging from 0.01 nM to 1.5 μM in 50 mM Tris-HCl, pH 7.5, in an 18 μL sample volume. After heating for 1.5 min at 90 °C, solutions were slowly cooled down to 25 °C, and 2 μL of 100 mM $MgCl_2$ was added to give a final concentration of 10 mM $MgCl_2$. The resulting solutions were then incubated at 25 °C for 18 h. Two microliters of 5% sucrose, 0.02% bromophenol blue, and xylene cyanol was added to each sample prior to loading onto a 15% polyacrylamide native gel. Electrophoresis and determination of K_d were performed as previously described (Fedor & Uhlenbeck, 1992). Equilibrium dissociation constants of E8C3U with substrates S8C, S8A, S8U, and S8G were measured by the same protocol except Pipes, pH 6.5, was used as a buffer.

Each cleavage rate constant report here is an average of at least three measurements which varied by less than 20%. Equilibrium dissociation constants (K_d) reported are also the average of three independent experiments with a standard deviation of less than 2-fold.

RESULTS

Two well-characterized hammerheads, HH8 and HH16, were used to examine the effects of changing the nucleotide at position 17 on the cleavage pathway. The sequences of

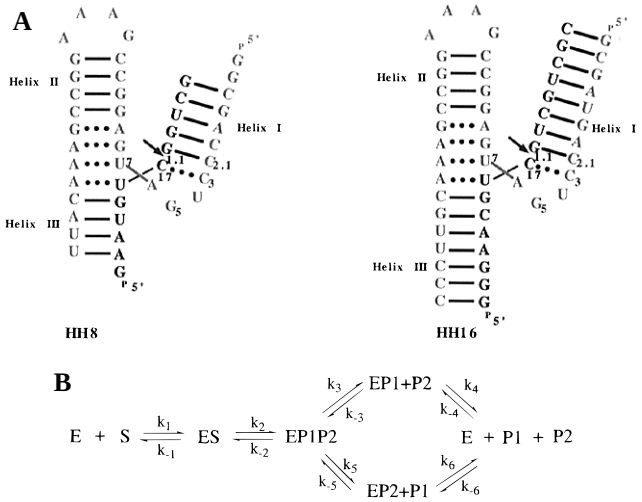

FIGURE 2: (A) Secondary structures of hammerhead 8 (HH8) and hammerhead 16 (HH16) arranged in a format that reflects the crystal structure. The stems and residues are numbered according to the standard nomenclature (Hertel et al., 1992). The cleavage site is indicated by the arrow. (B) Minimal kinetic mechanism for the intermolecular hammerhead cleavage reaction which consists of substrate (S) binding to the ribozyme (E), chemistry of the phosphodiester bond cleavage (k_2), and two pathways of product (P1, P2) release. The equilibrium dissociation constant (K_d) of the ribozyme−substrate complex (ES) and the internal equilibrium (K_{eq}^{int}) between the ES and the ribozyme−product complex (EP1P2) are equal to k_{-1}/k_1 and k_2/k_{-2} respectively.

both hammerheads (Figure 2A) are identical in their central core and in stem−loop II, but differ in the length and sequence of helix I and helix III that join the longer ribozyme oligonucleotide to the shorter substrate oligonucleotide. A minimal kinetic mechanism for intermolecular hammerhead cleavage (Figure 2B) involves the binding of the substrate oligonucleotide to the ribozyme through intermolecular base pairing, the reversible cleavage step, and two pathways for the release of the two products. The short stems of HH8 result in a rate of substrate dissociation (k_{-1}) that is much faster than the cleavage rate (k_2) (Fedor & Uhlenbeck, 1992). Consequently, the K_M of HH8 is equal to k_{-1}/k_1 or the K_d of substrate binding. HH16 has sufficiently long stems that the reaction products remain stably bound to the ribozyme after cleavage (Hertel et al., 1994). This permits determination of the reverse cleavage rate (k_{-2}) and the internal equilibrium constant ($K_{eq}^{int} = k_2/k_{-2}$) (Hertel & Uhlenbeck, 1995; Long et al., 1995). Since product binding is too weak in HH8 to obtain K_{eq}^{int} and substrate binding in HH16 is too tight to obtain K_d, it is not possible to use a single ribozyme−substrate pair to obtain data for both the substrate binding and cleavage equilibria. However, since the values of k_2 for both HH8 and HH16 are very similar to one another under a variety of buffer conditions (Dahm & Uhlenbeck, 1990; Dahm et al., 1993; Clouet-d'Orval et al., 1995; Hertel & Uhlenbeck, 1995; Stage et al., 1995), they are likely to react identically, making it reasonable to use them together to deduce the effects of the cleavage site nucleotide on the energetics of the reaction pathway. Most of the reactions were performed under "standard" hammerhead cleavage conditions of 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ at 25 °C.

Cleavage rates for the four N17 substrates of HH8 were determined by using a low concentration of ^{32}P-labeled substrate and a series of ribozyme concentrations. Values of k_{cat} and K_M for the C17, A17, and U17 substrates could be obtained from Eadie−Hofstee plots (Table 1). Both the k_{cat} (1.4 min^{-1}) and K_M (50 nM) values for the C17 substrate were in close agreement with those previously reported (Fedor & Uhlenbeck, 1992). The A17 substrate had only a slightly lower value of k_{cat} than the C17 substrate, but a 6-fold greater K_M. In contrast, the U17 substrate had a 5-fold slower k_{cat} than the C17 substrate, but the same value for K_M. The G17 substrate cleaved extremely slowly, making it impossible to obtain an accurate K_M value. At high concentrations of ribozyme, where the other three substrates are saturated, the cleavage rate of the G17 substrate of HH8 was 4×10^{-5} min^{-1}. Whereas cleavage of the G17 substrate is at the correct site, the rate is only about 10-fold faster than the rate of nonspecific RNA cleavage under these conditions (Hertel et al., 1996).

Table 1: Kinetic Parameters for HH8 Cleavage

residue 17	k_{cat} (min^{-1})	K_M (nM)
C	1.4	50
A	0.7	300
U	0.3	57
G	4.3×10^{-5} [a]	2 [b]

[a] Cleavage rate at 2 μM ribozyme. [b] K_d value obtained in Figure 3.

Two independent methods were used to obtain the binding affinity of the inactive G17 substrate to the ribozyme. In the first, the K_d was measured directly using nondenaturing gel electrophoresis (Pyle et al., 1990). A $K_d = 2.0$ nM was obtained for the G17 substrate by fitting data from a series of ribozyme concentrations (Figure 3A). In the second method, the inhibition of the rate of cleavage of the C17 substrate by the G17 substrate was determined. As shown in Figure 3B, the fraction of inhibition could be plotted as a function of competitor concentration and fit to a binding curve. The resulting value of $K_I = 1.9$ nM for the G17 substrate is in close agreement with the K_d determination. Thus, the hammerhead substrate with a G at the cleavage site is inactive, but binds the ribozyme more tightly than the active substrates.

In order to test whether the variability in the cleavage properties observed among the N17 substrates is due to the interaction of N17 with C3, the cytidine 3 residue in HH8 was mutated to a uridine (HH8C3U), and the cleavage properties of the four N17 substrates were investigated. Since the C3U hammerheads were not very active, it was not possible to determine accurate K_M values by using Eadie−Hofstee methods, so cleavage rates were determined at a saturating ribozyme concentration and binding constants were measured by native gel techniques. The rates of cleavage of the C, U, and A substrates with the C3U ribozyme (Table 2) were about 3000-fold less than the wild-type ribozyme; however, the relative order of cleavage was similar, with C and A somewhat faster than U. The G substrate remains nearly totally inactive. These data indicate that the differences in cleavage rates observed among the different N17 substrates are not due to the identity of C3, but that C3 has an important role in the cleavage mechanism.

The substrate binding affinities of the four N17 substrates to the C3U ribozyme were determined by using nondenaturing gels. As shown in Table 2, the C17 and G17 substrates bind the C3U ribozyme with affinities quite similar to those observed with the wild-type ribozyme. Presumably,

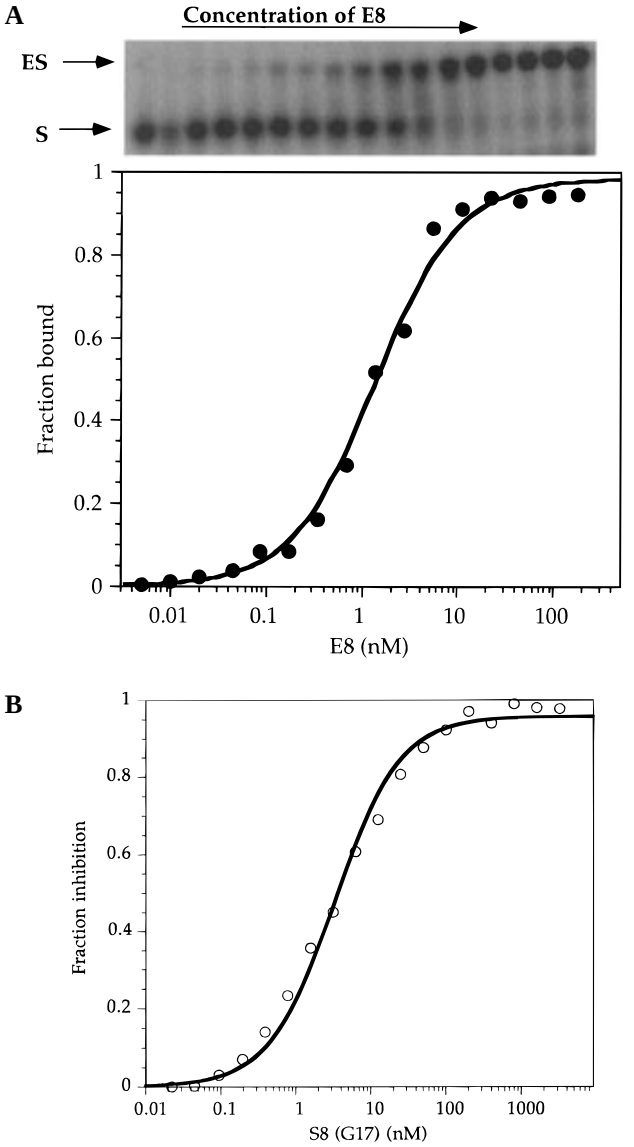

FIGURE 3: (A) Determination of the K_d of the E8·S(G17) complex by native gel electrophoresis. Autoradiogram of the gel separating labeled substrate from complex (top) is quantitated and fit to a simple binding equilibrium with a K_d = 2 nM (bottom). (B) Inhibition of HH8 by S(G17). At each S(G17) concentration, the cleavage rate (k_{obs}) of HH8 was determined and the fraction of inhibition ($I = 1 - (k_{obs}/k_2)$ calculated. The line assumes simple competitive inhibition with K_I = 1.9 nM.

Table 2: Cleavage Rates and Substrate Dissociation Constants of HH8C3U

residue 17	k_{obs}[a] (min^{-1})	K_d[b] (nM)
C	4.7×10^{-4}	40
A	3.8×10^{-4}	3
U	1.1×10^{-4}	2
G	2.6×10^{-5}	4

[a] Cleavage rate obtained at 2 μM ribozyme. [b] Measured with nondenaturing gels.

the continued tight binding of the G17 substrate is due to the formation of a G17−U3 pair, whereas any energy derived from a C3−C17 pair is not significantly different from that derived from a U3−C17 pair. In contrast, the A17 and U17 substrates bind the C3U ribozyme 100-fold and 30-fold more tightly than to the wild-type ribozyme. This additional binding energy is presumably due to the formation of the A17−U3 pair and the relatively stable U17−U3 mismatch

Table 3: Cleavage Rate and Internal Equilibrium Constant of HH16

residue 17	k_2[a] (min^{-1})	K_{eq}^{int}
C	0.9	100
A	0.4	120
U	0.07	120
G	1×10^{-5}	18[b]

[a] Rate at 1 μM ribozyme. [b] Estimated from the relative binding affinities of P1(G) and P1(C) (see text).

Table 4: Activation Parameters for Hammerhead 16 Variants

hammer-head	E_a (kcal/mol)	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ [cal/(mol·deg)]	$T\Delta S^{\ddagger}$ (kcal/mol)	$\Delta G^{\ddagger}$ (kcal/mol)
C17	18.3 ± 1.2	17.7 ± 1.0	−8.2 ± 1.5	−2.4 ± 0.4	20.1 ± 1.0
A17	19.3 ± 0.9	18.8 ± 0.8	−5.3 ± 1.0	−1.5 ± 0.3	20.3 ± 0.9
U17	22.1 ± 1.0	22.6 ± 1.1	0.6 ± 1.0	0.2 ± 0.3	21.4 ± 1.0

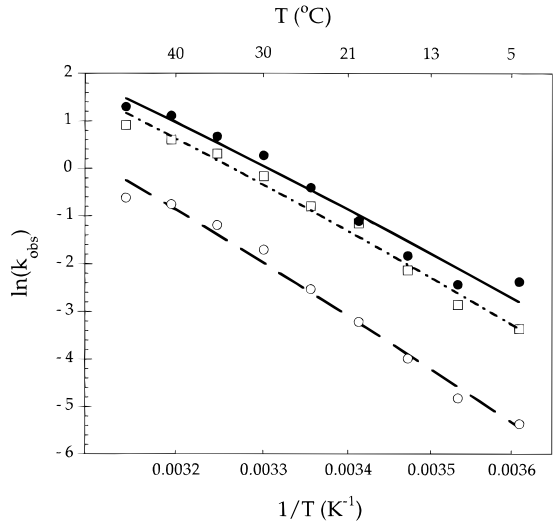

FIGURE 4: Temperature dependence of the rate constants of S16C, S16A, and S16U. Slopes of the Arrhenius plots of S16C (●), S16A (□), and S16U (○) give the values of activation energy, E_a, of the cleavage reactions (Table 4).

(SantaLucia et al., 1991; Baeyens et al., 1995; Wu et al., 1995; Wahl et al., 1996). Thus, although it appears quite possible to obtain binding energy of certain N17−N3 combinations, it is quite variable and shows little relation to the ability of the hammerhead to cleave.

The four N17 substrates of HH16 were used to verify the cleavage rate and determine the internal equilibrium. The cleavage rates were determined with trace ^{32}P-labeled substrate and a saturating concentration of ribozyme (Table 3). The cleavage rate for the C17 substrate (0.9 min^{-1}) is in good agreement with that obtained previously (Hertel et al., 1994). Although the absolute rates of all four HH16 substrates are slightly slower than for HH8, their relative rates are virtually the same, indicating that the effect of the cleavage site nucleotide is likely to be the same for both hammerheads.

One advantage of the longer helices in HH16 is that they permit measurement of the cleavage rate over a broader range of temperature than is convenient with HH8. Arrhenius plots (Figure 4) for the C, U, and A substrates of HH16 were obtained from data between 4 and 45 °C. These permit calculation of the values for $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ at 25 °C (Table 4). The values obtained for the C17 substrate are slightly different from those reported previously (Hertel & Uhlenbeck, 1995), but are believed to be more accurate since the

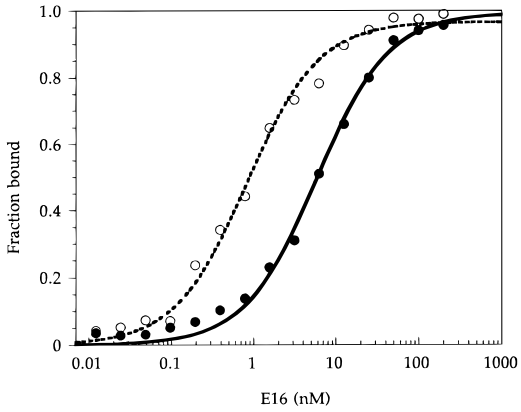

FIGURE 5: P1(C17) (●, solid line) and P1(G17) (○, dotted line) binding to E16·P2 determined by native gel electrophoresis. The lines are best fits to binding curves and correspond to K_d = 0.6 nM for P1(C17) and K_d = 0.08 nM for P1(G17).

buffer was adjusted to pH 7.5 at each temperature rather than correcting the cleavage rates for temperature-dependent pH changes of the buffer as was done previously. There is little difference between the activation parameters for the C17 and A17 substrates, and the slower U17 substrate is characterized by a greater $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$.

The internal equilibrium constants of the C, A, and U substrates were determined in reactions containing saturating ribozyme, a small excess of P2, and a trace concentration of each labeled P1. The internal equilibrium constant (K_{eq}^{int}) is the ratio of product to ligated substrate at equilibrium (Hertel et al., 1994; Hertel & Uhlenbeck, 1995). In all these cases, the value of K_{eq}^{int} was virtually identical, with the formation of products favored over substrate by a factor of about 100.

The slow cleavage rate of the G17 substrate makes it impossible to determine K_{eq}^{int} since nonspecific cleavage would occur during the 5 half-lives (240 days) needed to reach equilibrium. Since tighter substrate binding was observed with the G17 substrate of HH8, it was of interest to see whether tighter binding of the P1 product could be observed with HH16. A nondenaturing gel method was used to determine the affinity of P1 (C17) and P1 (G17) to the ribozyme−P2 complex. As shown in Figure 5, the affinity of the G17 product (K_d = 0.08 nM) was significantly more stable than the C17 product (K_d = 0.6 nM), suggesting that the G17−C3 pair also can form in the cleaved complex. Thus, P1 (G17) is only 1 kcal/mol more stable than P1 (C17) while the G17 substrate is 2 kcal/mol more stable than the C17 substrate (Table 1). This result suggests that ΔG_{int} for the G17 substrate is 1 kcal/mol less than the other three substrates, which would correspond to a K_{eq}^{int} = 18 instead of 100.

DISCUSSION

Two hammerheads with identical catalytic cores and different helix lengths were used to evaluate the effect of mutations at two critical positions in the molecule. This strategy was necessary because it is not possible to obtain both accurate substrate binding affinity and the rate of ligation with the same hammerhead. Since both hammerheads cleave at virtually the same rate in a variety of reaction conditions, they appear to be catalytically identical, so their joint use to study mutations is justified.

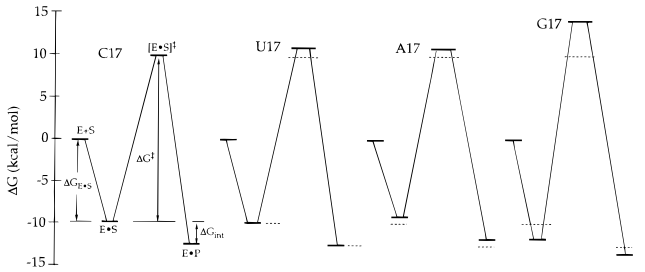

FIGURE 6: Composite free energy diagrams for hammerhead cleavage reactions with different nucleotides at position 17. A standard state of 1 M free substrate and ribozyme at 298 *K* is assumed to be the same for each hammerhead, $\Delta G_{E\cdot S} = -RT \ln K_{E\cdot S}$ for HH8, $\Delta G_{int} = -RT \ln K_{eq}^{int}$ for HH16, and $\Delta G^{\ddagger} = -RT \ln (kh/k_BT)$ where k is the average of k_2 values for HH8 and HH16. Dotted lines represent the substrate which contains C at the cleavage site.

A useful way to summarize the hammerhead reaction pathway is with a free energy diagram of the reaction coordinate. Since the data were obtained using two different hammerheads, the diagrams for each N17 mutation shown in Figure 6 are composites. They summarize three measurements of free energy changes that relate the different states in the reaction pathway. The free energy of substrate binding, $\Delta G_{E\cdot S}$, is obtained from the K_M or K_d of HH8 mutants. The free energy difference between uncleaved and cleaved hammerhead, ΔG_{int}, is obtained from the internal equilibrium of HH16 mutants. Finally, the free energy of the transition state, $\Delta G^{\ddagger}$, is calculated from the average of the very similar k_2 values of the two hammerheads. It is striking that each N17 mutation affects the reaction pathway quite differently. If the C17 hammerhead is used as a basis of comparison, the U17 mutation increases the transition state energy by about 1 kcal/mol, but has no effect on substrate binding or the internal equilibrium. In contrast, the A17 substrate binds weaker than the C17 substrate, but the relative free energy of the transition state and the product remains the same for both. The G17 mutation is the most complicated since it shows about 2 kcal/mol tighter binding and a 4.3 kcal/mol higher transition state. It was not possible to obtain a ΔG_{int} for G17, but it can be estimated from the binding of P1 to E·P2 and is perhaps slightly different than the others. This involved picture of the consequences of mutating a single position with different residues is not unexpected for a biochemical catalyst. Similar results are often observed with protein enzymes. For example, when the identity of the amino acid adjacent to the cleavage site of collagenase is modified, either K_M or k_{cat} is changed, depending upon the amino acid that is inserted (Tsu & Craik, 1996). Similarly, different mutations of an active site residue in thymidylate synthase can perturb K_M or k_{cat} differently (Carreras & Santi, 1995). The results clearly emphasize the importance of examining a spectrum of mutations and evaluating the complete pathway before activity data are related to a structure.

Both available X-ray crystal structures of the hammerhead are enzyme−inhibitor complexes since the attacking 2′-hydroxyl has been altered to either a hydrogen (Pley et al., 1994) or a 2′-*O*-methyl (Scott et al., 1995). The assumption that these structures closely resemble an E·S complex (McKay, 1996) has recently been supported by the observation that an all-RNA version of one of the hammerheads has an identical structure when crystallized in the absence

of divalent ion (W. Scott, personal communication). Thus, the value of $\Delta G_{E\cdot S}$, the energy of formation of the E·S complex, is expected to be the parameter most easily correlated with the crystal structure. $\Delta G_{E\cdot S}$ reflects the energy of formation of helices I and III as well as any energetic contribution of formation of the catalytic core, including the C3−C17 interaction.

Although it is difficult to account for all the factors that contribute to the magnitude of $\Delta G_{E\cdot S}$, it is easier to interpret differences in $\Delta G_{E\cdot S}$ between the eight N3−N17 combinations that were tested. Since the most stable combinations involved either a base pair (C3−G17 and U3−A17), a wobble pair (U3−G17), or a mismatched pair that is reported to be quite stable (U3−U17) (SantaLucia et al., 1991; Baeyens et al., 1995; Wu et al., 1995; Wahl et al., 1996), it seems clear that a certain amount of stabilization energy may be contributed by the N3−N17 interaction. However, the C3−C17 combination present in the crystal structure, as well as the U3−C17 and C3−U17 combinations, bound with affinities about 1 kcal/mol weaker than the four most stable pairs. The only available thermodynamic data that can be used to put these results into any context are the values for the free energy contribution of various base combinations at the end of an RNA helix (Freier et al., 1986). Although such terminal pairs are likely to be under quite different thermodynamic constraints than the N3−N17 pair in the hammerhead, it is interesting that a terminal base pair will stabilize the adjacent helix by 2−3 kcal/mol, while the C−C, U−C, and C−U mismatches stabilize the helix by about 1 kcal/mol. Thus, the binding increments in the hammerhead do not seem to be very different than a terminal pair.

If the absolute contribution of the C3−C17 interaction to $\Delta G_{E\cdot S}$ is indeed in the neighborhood of 1 kcal/mol, the energetic cost of disrupting this interaction to reach an active conformation is not very large. Thus, even a mechanism as extreme as that proposed by Pley et al. (1994), where C17 is rotated nearly 180° from its position in the crystal structure, cannot be excluded on thermodynamic grounds. This model not only results in an *in-line* configuration of the attacking and leaving oxygens but also places the cleavage site in proximity to C3, a residue that is clearly important for catalysis. Other models which involve less extreme disruptions of the C3−C17 interaction (Scott et al., 1995; Setlik et al., 1995) are, of course, also compatible with the data.

The exceptionally weak binding of the C3−A17 pair deserves special mention. Since $\Delta G_{E\cdot S}$ for this is more than 2 kcal/mol weaker than either of the N3−N17 base pairs and weaker than expected for a terminal mismatch, it appears that this combination is particularly detrimental in the hammerhead structure. Perhaps the larger adenosine ring cannot be accommodated in position 17 without forming a disruptive contact with C3 or a neighboring functional group. As a result of this potential interaction, the position of A17 may be shifted to an environment where less stabilization energy is available.

In general, changing the residue at position 17 affects the cleavage rate quite differently than it affects substrate binding. When the U17 substrate is compared to the C17 substrate, there is no difference in the substrate binding energy, but the cleavage rate is 5−10-fold slower. This finding suggests that the cytidine ring can stabilize the transition state in a way that uridine cannot. The availability of additional binding energy in the hammerhead transition state has been proposed based upon a kinetic and thermodynamic analysis of a series of truncated variants of HH16 (Hertel et al., 1996). Presumably, the 1 kcal/mol difference in binding energy in the transition state is due to the nitrogen-3 and exocyclic amino functional groups of cytidine as well as its comparatively larger dipole. The precise identification of all the functional groups in the cytidine ring responsible for transition state stabilization awaits experiments with modified nucleotides at this position.

The cleavage properties of the G17 substrate are more complicated. As discussed above, the ground state of the G17 substrate is stabilized about 2 kcal/mol by the proposed formation of a G17−C3 pair. However, the cleavage rate of the G17 substrate is much too slow to simply be explained by the need to break this pair before the transition state is reached. As shown in Figure 6, the transition state of the G17 substrate is about 3 kcal/mol higher in energy than that of C17 and not far from the transition state of the uncatalyzed rate of cleavage. Presumably, the guanosine is unable to make all the contacts necessary to stabilize the transition state, or it is more difficult to reach the transition state structure due to a steric constraint.

The A17 substrate is perhaps the most interesting. The 1 kcal/mol weaker substrate binding does not result in a faster cleavage rate. In contrast to U17 and G17, the cleavage rate of the A17 substrate is essentially the same as C17. These results suggest that both the ground state and transition state structures of the A17 substrate are not the same as the C17 substrate. In other words, the reaction pathway of the A17 substrate is thermodynamically different from the C17 substrate. X-ray crystallographic and NMR experiments will be useful to determine whether any structural differences exist in the ground state. An analysis of the cleavage rate of purine analogues may be informative in identifying the functional groups responsible for the large differences between the A and G reactions.

The free energy term, ΔG_{int}, reflects thermodynamic differences between the E·S and E·P complexes and, for the C17 substrate, greatly favors cleavage. Several different factors are believed to contribute to ΔG_{int} including a change in the relative affinity of one or more metal ions to the two states and an increased flexibility after cleavage (Hertel & Uhlenbeck, 1995; Long et al., 1995). Interestingly, despite large effects in the binding and catalysis steps, there is little or no effect of changing the cleavage site nucleotide on ΔG_{int}. In the case of C, U, and A, where ΔG_{int} can be obtained directly, it remains the same. The value of ΔG_{int} for the G17 variant appears to favor ligation somewhat more than the others, but the value is obtained indirectly, and this may lead to increased error in the value. Therefore, it appears that metal ion binding, flexibility, and other factors that influence ΔG_{int} may not involve the residue at position 17.

In summary, kinetic and thermodynamic methods were applied to investigate the effects of hammerhead mutations on the cleavage reaction. In the current study, only three mutations at a single nucleotide position were examined. However, this approach is applicable to any mutation in the hammerhead. It is clear from the limited data that the structural requirements of the substrate binding, catalysis, and internal equilibrium steps are quite different. In order to relate the data to the crystal structure of the hammerhead, with the ultimate goal of understanding the cleavage mech-

anism, more sophisticated atomic substitution of the cleavage site nucleotide will be needed, together with additional structural studies.

ACKNOWLEDGMENT

We wish to thank June Gustafson for assistance in preparing the manuscript.

REFERENCES

Adams, C. J., Murray, J. B., Arnold, J. R. P., & Stockley, P. G. (1994) *Tetrahedron Lett. 35*, 1597−1600.
Baeyens, K. J., De Bondt, H. L., & Holbrook, S. R. (1995) *Struct. Biol. 2*, 56−62.
Buzayan, J. M., Gerlach, W. L., & Bruening, G. (1986) *Nature 323*, 349−353.
Carreras, C. W., & Santi, D. V. (1995) *Annu. Rev. Biochem. 64*, 721−762.
Clouet-d'Orval, B., Stage, T. K., & Uhlenbeck, O. C. (1995) *Biochemistry 34*, 11186−11190.
Dahm, S. C., & Uhlenbeck, O. C. (1990) *Biochimie 72*, 819−823.
Dahm, S. C., Derrick, W. B., & Uhlenbeck, O. C. (1993) *Biochemistry 32*, 13040−13045.
Fedor, M. J., & Uhlenbeck, O. C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1668−1672.
Fedor, M. J., & Uhlenbeck, O. C. (1992) *Biochemistry 31*, 12042−12054.
Freier, S. M., Kierzek, R., Jaeger, J. A., Sugimoto, N., Caruthers, M. H., Neilson, T., & Turner, D. H. (1986) *Proc. Nat. Acad. Sci. U.S.A. 83*, 9373−9377.
Hertel, K. J., & Uhlenbeck, O. C. (1995) *Biochemistry 34*, 1744−1749.
Hertel, K. J., Pardi, A., Uhlenbeck, O. C., Koizumi, M., Ohtsuka, E., Uesugi, S., Cedergren, R., Eckstein, F., Gerlach, W. L., Hodgson, R., & Symons, R. H. (1992) *Nucleic Acids Res. 20*, 3252.
Hertel, K. J., Herschlag, D., & Uhlenbeck, O. C. (1994) *Biochemistry 33*, 3374−3385.
Hertel, K. J., Herschlag, D., & Uhlenbeck, O. C. (1996) *EMBO J.* (in press).
Hutchins, C. J., Rathjen, P. D., Forster, A. C., & Symons, R. H. (1986) *Nucleic Acids Res. 14*, 3627−3640.
Koizumi, M., & Ohtsuka, E. (1991) *Biochemistry 30*, 5145−5150.
Koizumi, M., Iwai, S., & Ohtsuka, E. (1988a) *FEBS Lett. 239*, 285−288.
Koizumi, M., Iwai, S., & Ohtsuka, E. (1988b) *FEBS Lett. 228*, 228−230.
Koizumi, M., Hayase, Y., Iwal, S., Kamiya, H., Inoue, H., & Ohtsuka, E. (1989) *Nucleic Acids Res. 17*, 7059−7071.
Long, D. M., LaRiviere, F. J., & Uhlenbeck, O. C. (1995) *Biochemistry 34*, 14435−14440.
McKay, D. B. (1996) *RNA 2*, 395−403.
Milligan, J. F., Groebe, D. R., Witherell, G. W., & Uhlenbeck, O. C. (1987) *Nucleic Acids Res. 15*, 8783−8798.
Nakamaye, K. L., & Eckstein, F. (1994) *Biochemistry 33*, 1271−1277.
Perreault, J. P., Wu, J., Coussineau, B., Ogilvi, K. K., & Cedegren, R. (1990) *Nature 344*, 565−567.
Perriman, R., Delves, A., & Gerlach, W. L. (1992) *Gene 113*, 157−163.
Pley, H. W., Flaherty, K. M., & McKay, D. B. (1994) *Nature 372*, 68−74.
Prody, G. A., Bakos, J. T., Buzayan, J. M., Schneider, I. R., & Bruening, G. (1986) *Science 231*, 1577−1580.
Pyle, A. M., McSwiggen, J. A., & Cech, T. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8187−8191.
Ruffner, D. E., Stormo, G. D., & Uhlenbeck, O. C. (1990) *Biochemistry 29*, 10695−10702.
SantaLucia, J., Jr., Kierzek, R., & Turner, D. H. (1991) *Biochemistry 30*, 8242−8251.
Scaringe, S. A., Francklyn, C., & Usman, N. (1990) *Nucleic Acids Res. 18*, 5433−5441.
Scott, W., Finch, J., & Klug, A. (1995) *Cell 81*, 991−1002.
Setlik, R. F., Shibata, M., Sarma, R. H., Sarma, M. H., Kazim, A. L., Ornstein, R. L., Tomasi, T. B., & Rein, R. (1995) *J. Biomol. Struct. Dyn. 13*, 515−522.
Shimayama, T., Nishikawa, S., & Taira, K. (1995) *Biochemistry 34*, 3649−3654.
Slim, G., & Gait, M. J. (1991) *Nucleic Acids Res. 19*, 1183−1188.
Stage, T. K., Hertel, K. J., & Uhlenbeck, O. C. (1995) *RNA 1*, 95−101.
Tsu, C. A., & Craik, C. S. (1996) *J. Biol. Chem. 271*, 11563−11570.
Uhlenbeck, O. C. (1987) *Nature 328*, 596−600.
Usman, N., Ogilvie, K. K., Jiang, M.-Y., & Cedergren, R. J. (1987) *J. Am. Chem. Soc. 109*, 7845−7854.
van Tol, H., Buzayan, J. M., Feldstein, P. A., Eckstein, F., & Bruening, G. (1990) *Nucleic Acids Res. 18*, 1971−1975.
Wahl, M. C., Rao, S. T., & Sundaralingam, M. (1996) *Struct. Biol. 3*, 24−31.
Wu, M., McDowell, J. A., & Turner, D. H. (1995) *Biochemistry 34*, 3204−3211.
Zoumadakis, M., & Tabler, M. (1995) *Nucleic Acids Res. 23*, 1192−1196.

BI962165J