Kinetic characterization of two I/II format hammerhead ribozymes

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ABSTRACT

Five new hammerhead ribozymes were designed that assemble through the formation of helices I and II (I/II format) instead of the more standard assembly through helices I and III (I/III format). The substrate binding and cleavage properties of such hammerheads could potentially be different due to the absence of loop II and the requirement for the entire catalytic core to assemble. Two I/II format hammerheads, HHα1 and HHα5, which show structural homogeneity on native gels, were characterized kinetically. The association rate constants of both I/II hammerheads are unusually slow compared to the rate of RNA duplex formation. The dissociation rate constants indicate that the hammerhead core destabilizes an uninterrupted RNA helix somewhat less than was observed for I/III hammerheads. Whereas the cleavage rate constant of HHα5 is similar to that observed for I/III hammerheads, HHα1 cleaves 10-fold faster than any hammerhead previously reported. The temperature and pH dependence of the cleavage rate constant of HHα1 are similar to those reported for I/III hammerheads, suggesting a similar mechanism of cleavage.

Keywords: hammerhead; kinetics; substrate binding; catalytic RNA

INTRODUCTION

The hammerhead ribozyme is found naturally as an intramolecular self-cleaving motif, but it is usually studied biochemically as a bimolecular reaction in which two oligoribonucleotides assemble into the active hammerhead through the formation of two RNA helices. Because the hammerhead secondary structure contains three helices, three different bimolecular formats are possible. In the commonly studied I/III format, a small substrate oligonucleotide binds to the larger ribozyme by forming helix I and helix III (Haseloff & Gerlach, 1988). In the I/II format, the hammerhead is assembled by combining oligonucleotides of approximately similar size through the formation of helices I and II (Uhlenbeck, 1987). Finally, the rarely studied II/III format uses a small ribozyme fragment and a large substrate (Koizumi et al., 1988; Jeffries & Symons, 1989). It has generally been accepted that the format in which the hammerhead is assembled does not greatly affect its structure or function. All three formats are active and show similar rates of cleavage under similar reaction conditions. In addition, the crystal structure of a I/III

hammerhead (Pley et al., 1994) is virtually identical to that of a I/II hammerhead (Scott et al., 1995).

Primarily because of potential application for gene inactivation (Haseloff & Gerlach, 1988), the I/III hammerheads have been subjected to rigorous kinetic analysis. A minimal kinetic mechanism has been established, consisting of assembly, cleavage, and product release steps, and most (Fedor & Uhlenbeck, 1992) or all (Hertel et al., 1994) of the elemental rate constants have been determined for four substantially different I/III hammerheads. Although the complex rate constants, k_{cat} and K_M, have been reported for two different I/II hammerheads (Uhlenbeck, 1987; Koizumi & Ohtsuka, 1991), the corresponding elemental rate constants have not been determined. There are a number of reasons why more extensive kinetic characterization of hammerheads in the I/II format would be desirable. First of all, format I/II hammerheads are very convenient for detailed structure–function studies at the atomic level because both oligonucleotides are in the size range that permits convenient chemical synthesis. Secondly, during the catalytic cycle of I/II hammerheads, the entire catalytic core must form prior to cleavage and break upon product release. In contrast, NMR evidence from a I/III hammerhead suggests that much of the catalytic core is formed in the ribozyme oligonu-

cleotide and does not change substantially upon binding of substrate (Legault, 1995). Thus, it is possible that analysis of the elemental rate constants of I/II hammerheads may provide information about the assembly of the catalytic core. Finally, despite the similar crystal structures, it remains possible that the difference in the format could affect the cleavage reaction in some unanticipated way. For example, positive or negative interactions between the backbones of loop II and the nearby stem I in I/III hammerheads may be absent in a I/II hammerhead, resulting in changes in one or more rate constants.

A major complication in the kinetic characterization of ribozyme cleavage reactions is the propensity of oligonucleotides to adopt multiple conformations that lead to kinetic inhomogeneity. This problem greatly complicated the analysis of several I/III hammerheads (Fedor & Uhlenbeck, 1992) and is also expected to be severe in I/II hammerheads. Thus, before kinetic characterization of a I/II hammerhead can begin, a search for a kinetically well-behaved ribozyme–substrate pair must be made.

RESULTS

Five new hammerhead sequences in the I/II format (Fig. 1) were designed to resemble HH8, a well-characterized I/III format hammerhead that has helices that are sufficiently short that the rates of substrate dissociation and product release are faster than the cleavage rate (Fedor & Uhlenbeck, 1990, 1992; Dahm et al., 1993). The new hammerheads HHα1 to HHα5 (Fig. 1) contained helix I sequences that were either identical to helix I of HH8 (HHα5) or had a similar thermodynamic stability. The helix II sequences of HHα1 to HHα5 had thermodynamic stabilities similar to helix III of HH8, but all contained the G-C base pair that is part of the catalytic core in helix II.

Previous experiments with I/III hammerheads have revealed that when either the ribozyme or the substrate oligonucleotide appeared to present multiple conformations on a nondenaturing gel, the cleavage reaction either showed kinetic inhomogeneity or the K_M values were very high, indicating poor substrate binding (Fedor & Uhlenbeck, 1992). Thus, the conformational homogeneity of the 10 oligonucleotides comprising the substrates and ribozymes of HHα1 to HHα5 were examined on nondenaturing gels in buffer conditions similar to those used for cleavage reactions. As a control, the substrate and ribozyme of the O1/O2 hammerhead (Uhlenbeck, 1987) were also examined. Thermal denaturation studies on both O1 and O2 indicated that these oligomers formed substantial secondary structure (Ruffner et al., 1989). In each case, oligonucleotide concentrations that span the range typically used in kinetic experiments (5–5,000 nM) were tested. Data for the ribozyme oligonucleotides α1 to α3 are shown in Fig-

ure 2. In the case of α1, a single band was observed at all concentrations, whereas α2 and α3 showed additional slower migrating species at high concentrations, indicating the presence of aggregation states. Table 1 summarizes the results for the other oligonucleotides. Of the 12 oligonucleotides tested, O1, O2, and the ribozymes of HHα2, HHα3, and HHα4 showed multiple species on native gels. Although a single species on a nondenaturing gel does not guarantee homogeneous kinetic properties, multiple species are clearly detrimental to straightforward kinetic analysis. Thus, only HHα1 and HHα5 were chosen for further kinetic characterization.

The rates of cleavage for HHα1 and HHα5 were determined with trace concentrations (<2 nM) of substrate and a series of ribozyme concentrations between 10 nM and 10 μM. Experiments were carried out in "standard" hammerhead reaction conditions of 50 mM buffer pH 7.5, 10 mM MgCl$_2$ at 25 °C. However, in the case of HHα1, it became evident that at high ribozyme concentrations, the cleavage rate became too fast to be measured precisely by manual pipetting methods. Thus, data for this hammerhead were collected at pH 6.5, where the reaction rate was 10-fold slower (Dahm et al., 1993). For both hammerheads, cleavage rates at every ribozyme concentration can be described by a single rate constant, and the extent of cleavage at long incubation times is greater than 90%, suggesting that both substrates are kinetically homogeneous. In each case, the cleavage rate saturates and the data fit Michaelis–Menten behavior. The data for HHα1 are shown in Figure 3. The rate of cleavage at subsaturating ribozyme concentration gives the apparent second-order rate constant k_{cat}/K_M. The kinetic properties of both hammerheads deduced from the ribozyme excess experiments are summarized in Table 2. The data for the two hammerheads were quite different with HHα1, showing a lower K_M and a remarkably fast k_{cat} in the pH 6.5 buffer.

Cleavage reactions for both hammerheads were analyzed with a 10-fold excess of substrate and at near-saturating ribozyme concentration. As illustrated in Figure 4A, HHα5 shows an inflection in the reaction

TABLE 1. Summary of the structural homogeneities of the substrate (S) and ribozyme (E) RNAs deduced by 15% nondenaturing gel electrophoresis.

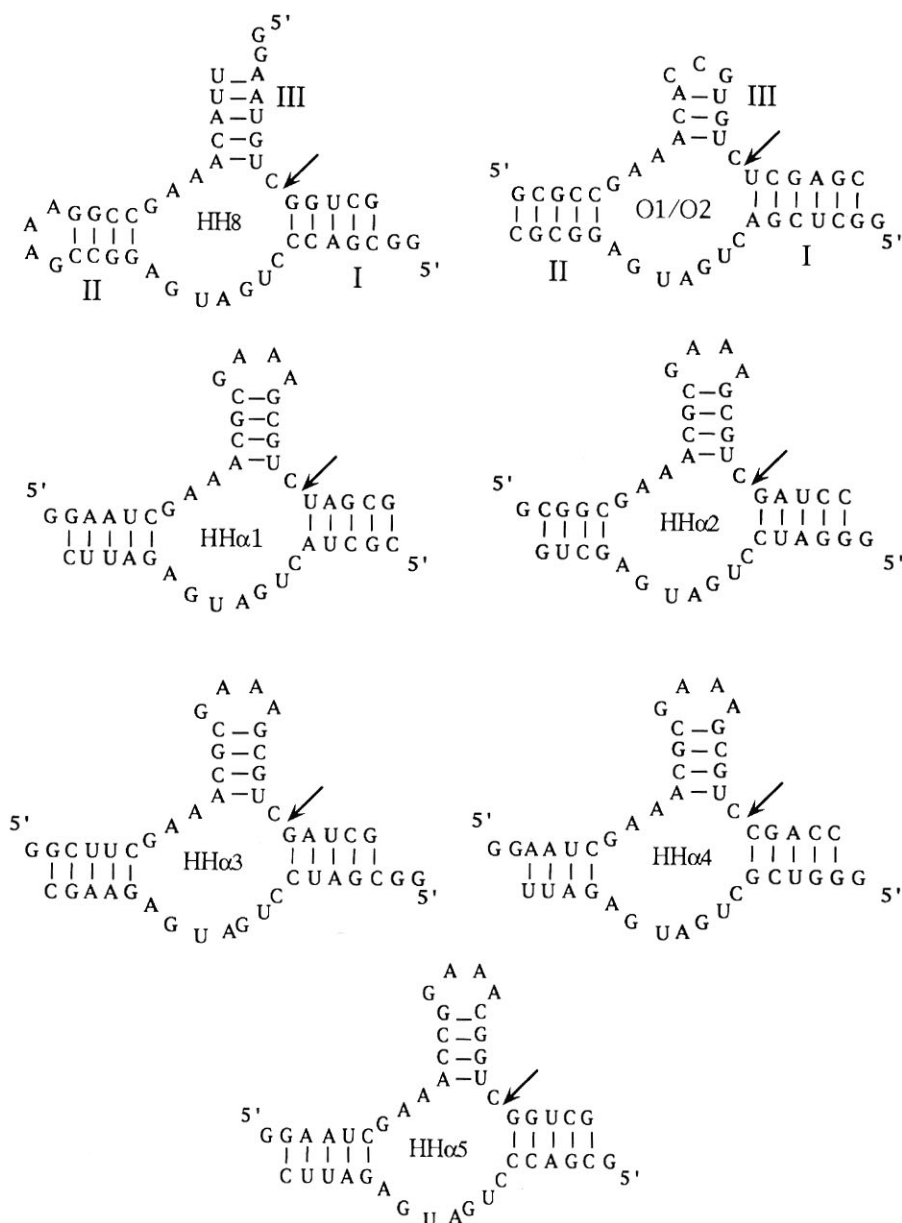| Single species	Multiple species
Eα1	Eα2
Eα5	Eα3
Sα1	Eα4
Sα2	O1
Sα3	O2
Sα4	
Sα5	

FIGURE 1. Sequences of a I/III format hammerhead (HH8), the previously studied I/II format hammerhead (O1/O2), and five new I/II format hammerheads (HHα1 to HHα5). Helices I, II, and III are indicated on HH8 and O1/O2. In each case, the oligonucleotide that is cleaved is defined as the substrate (S) and the other oligonucleotide as the ribozyme (E). The 5′ cleavage product is defined as P1 and the 3′ cleavage product as P2. Thus, HHα5 consists of Eα5 and Sα5 and cleaves to give P1α5 and P2α5.

progress curve that can be described by an initial rate of 0.5 min^{-1} and a steady-state rate of 0.1 min^{-1} at pH 7. Extrapolating the steady-state rate to zero time indicates that the faster initial rate is associated with the first turnover. Such "burst" behavior suggests that the rate of release of one of the two products of HHα5 is somewhat slower than the cleavage rate. When a derivative of the ribozyme that lacks the 3′ terminal C residue was used in the same experiment, an identical burst was observed (data not shown). Because removing this residue would be expected to destabilize P1 binding and no effect on the burst was seen, the data suggest that the release of the 3′ product P2 is the rate-limiting step in the multiple turnover reaction.

A similar saturating multiple turnover experiment carried out with HHα1 (Fig. 4B) shows no indication of an initial "burst," suggesting that in this case the cleavage rate is slower than product release for this hammerhead. This result is consistent with the fact that HHα1 only differs from HHα5 by having a less stable helix I, so P2 release is expected to be faster. When the cleavage rate was measured at pH 6.5 for a series of substrate concentrations ranging from 10 nM to 5 μM combined with one-tenth the concentration of ribozyme, the resulting kinetic parameters $k_{cat} = 1.2 \pm 0.4$ min^{-1}, $K_M = 380 \pm 90$ nM, and $k_{cat}/K_M = 2.5 \pm 0.2$ μM^{-1} min^{-1} agreed very well with the ribozyme excess parameters in Table 2.

Further examination of the kinetic properties of these hammerheads involves determining the association and dissociation rate constants of the following kinetic framework:
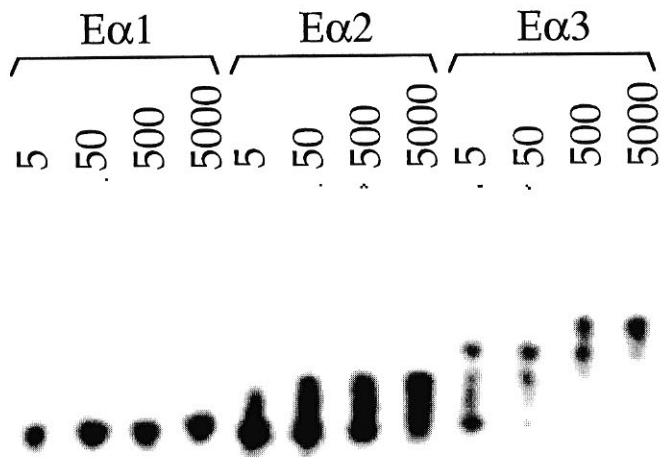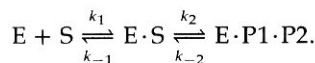
FIGURE 2. Test of the structural homogeneity of Eα1, Eα2, and Eα3 at the indicated ribozyme concentrations (nM) using 15% nondenaturing gel electrophoresis.

FIGURE 3. Eadie–Hofstee plot of HHα1, $K_M = 370 \pm 50$ nM, $k_{cat} = 1.8 \pm 0.3$ min^{-1} at pH 6.5.

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

The substrate dissociation rate constant, k_{-1}, was determined by a pulse–chase experiment where the chase was done by dilution and not by addition of an excess of substrate (Fedor & Uhlenbeck, 1992; Hertel et al., 1994; Werner & Uhlenbeck, 1995). In the case of HHα1, a saturating concentration of ribozyme (2 μM) and a trace amount of radioactive substrate were mixed for 7 s at pH 6.5 to complete binding, and then diluted 200-fold. The cleavage was measured during the chase period. A parallel reaction was performed without the dilution. In addition, a control reaction diluted immediately showed no cleavage, indicating that the dilution was sufficient to prevent reassociation of the substrate to the ribozyme. As shown in Figure 5, some but not all of the substrate is converted to product during the chase period, indicating that the substrate dissociation rate is similar to the cleavage rate. From these data, the rate constant for substrate dissociation, k_{-1}, can be calculated from the fraction of substrate that goes on to cleave after the initial binding period (Fedor & Uhlenbeck, 1992). The k_{-1} value obtained was 0.4 ± 0.2 min^{-1} at pH 6.5. This experiment was repeated at pH 6.0, where the cleavage rate was eightfold slower, and at

pH 7.0, where the cleavage rate was fivefold faster. As expected, a smaller fraction of the substrate (35%) was cleaved after the chase at pH 6.0 and a larger fraction (60%) at pH 7.0. The calculated values of k_{-1} from these experiments were 0.4 ± 0.2 min^{-1} and 1.2 ± 0.5 min^{-1}, respectively. Similar experiments were carried out with HHα5 in conditions adjusted for the properties of this hammerhead. A saturating ribozyme con-

FIGURE 4. Hammerhead kinetics during the approach to steady state. **A:** HHα5 with 2 μM ribozyme and 20 μM substrate at pH 7. **B:** HHα1 with 200 nM ribozyme and 2 μM substrate at pH 6.

TABLE 2. Kinetic parameters of I/II format hammerheads.[a]

Hammerhead	k_{cat}/K_M (μM^{-1} min^{-1})	k_{cat} (min^{-1})	K_M (μM)
HHα5	1.7 ± 0.3	2.5 ± 0.5	0.9 ± 0.2
HHα1	2.7 ± 0.6	1.8 ± 0.3	0.37 ± 0.10

[a] Experiments were performed in ribozyme excess at pH 7.5 for HHα5 and at pH 6.5 for HHα1.
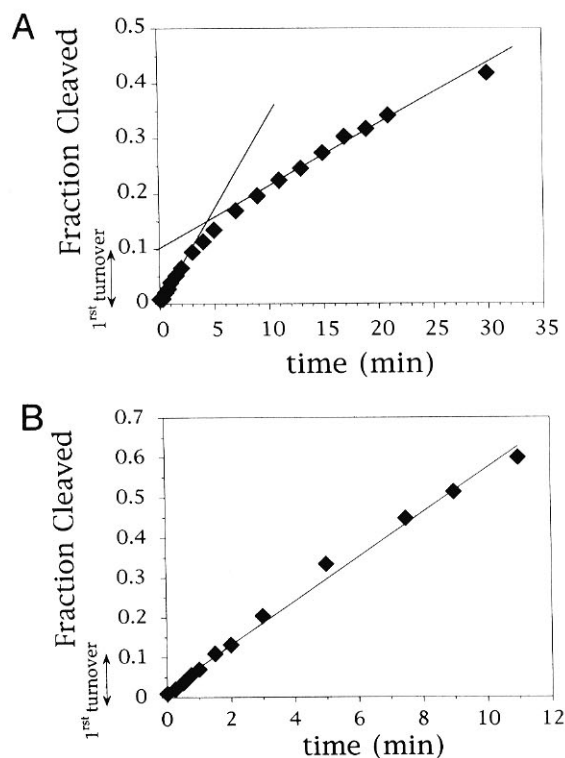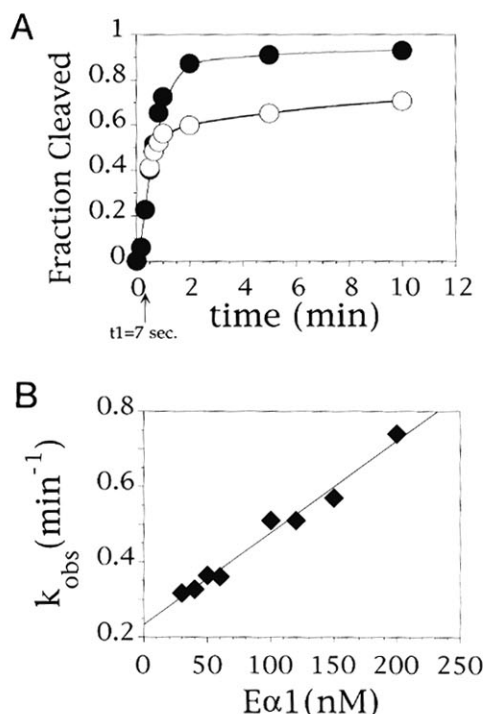
FIGURE 5. A: Pulse–chase experiment to determine the rate of substrate of dissociation, k_{-1}, for HHα1 at pH 6.5. The reaction was diluted 200-fold at $t_1 = 7$ s [diluted (O) or not diluted ($\bullet$)]. The fraction of substrate cleaved between t_1 and the end of the chase, $F_{chase} = 0.57$. The fraction of substrate cleaved without the chase, $F_{control} = 0.93$. The rate of dissociation k_{-1} is calculated from the relationship $F_{chase}/F_{control} = k_2/(k_2 + k_1)$ (Fedor & Uhlenbeck, 1992). In this case, $k_{-1} = 0.4 \pm 0.2$ min^{-1}. B: Rate of substrate binding, k_1, for HHα1 at pH 6.5. Values of k_{obs} were obtained in pulse–chase experiments at various ribozyme concentrations. Data fit to $k_{obs} = k_1[E] + k_{-1}$ give $k_1 = 2.4 \pm 0.5$ μM^{-1} min^{-1} and $k_{-1} = 0.23 \pm 0.90$ min^{-1}.

centration (10 μM) was mixed with a trace amount of radioactive substrate for 45 s at pH 6.5 before 200-fold dilution. The rate constant for substrate dissociation, k_{-1}, was calculated from these data to be 0.035 ± 0.015 min^{-1}.

The association rate constant of the substrate to the ribozyme for HHα1 was measured by using a pulse–chase method (Fedor & Uhlenbeck, 1992; Hertel et al., 1994) where the chase was done by dilution. A given subsaturating ribozyme concentration was mixed with a trace amount of labeled substrate for several times, t_1, and then diluted 200-fold to prevent further binding. The diluted mixture was allowed to cleave for 1 h to ensure that every labeled substrate that had bound to the ribozyme during t_1 was either converted to product or dissociated. The amount of product formed as a function of time t_1, k_{obs}, describes the rate of approach to equilibrium of the binding of the ribozyme to the substrate and is the sum of the rate constants of the forward and reverse reactions (Herschlag & Cech, 1990; Hertel et al., 1994):

$$k_{obs} = k_1[E] + k_{-1}.$$

Thus, k_{obs} was determined at a number of ribozyme concentrations, and a plot of k_{obs} versus ribozyme concentration gives the rate constant $k_1 = 2.4 \pm 0.5$ μM^{-1} min^{-1} from the slope and $k_{-1} = 0.23$ min^{-1} from the intercept (Fig. 5B).

For HHα5, the association rate constant, k_1, was obtained in two different ways. First, the experimentally determined values of k_{-1} and K_M values can be used to calculate $k_1 = 2.2$ μM^{-1} min^{-1}. Second, the experimental value for the observed second-order rate constant obtained at subsaturating substrate concentration of 1.7 ± 0.3 μM^{-1} min^{-1} is in good agreement. The values for the association and dissociation rate constants of HHα1 and HHα5 are summarized in Table 3.

For HHα1, the experimentally determined values of k_{-1} and k_1 can be used to calculate a Michaelis constant $K_M = (k_{-1} + k_2)/k_1 = 0.9 \pm 0.3$ μM at pH 6.5. Considering the standard deviation error in the measurement of the individual rates, this value agrees reasonably well with the experimentally determined value of $K_M = 0.37 \pm 0.10$ μM reported in Table 2. For HHα1, $K_M \simeq K_d$ at low pH values where k_2 is slow, but K_M is slightly greater than K_d at higher pH values.

To further characterize HHα1 and HHα5, the effects of extrinsic parameters on the rate of cleavage were examined. The pH dependence of the rate of cleavage of HHα1 was determined at a ribozyme concentration of 1 μM and trace labeled substrate with 10 mM Mg^{2+} in 50 mM sulfonate buffer (Fig. 6A). The experiments were carried out at 4 °C because the rapid cleavage rate made it difficult to obtain accurate data at 25 °C at the higher pH values. Similar to several I/III hammerheads, the log of the cleavage rate increases linearly with pH from pH 5.7 to 7.5, giving a slope of 1.1, indicating that a single deprotonation occurred.

The rate of cleavage for HHα1 was measured as a function of temperature ranging from 2 to 25 °C at 1 μM ribozyme and trace substrate at pH 6.5. At higher temperatures, the binding of ribozyme to substrate became much weaker, making it difficult to achieve saturation at reasonable concentrations. Between 4 and 25 °C, a linear van't Hoff plot is observed (Fig. 6B) that gives an activation energy of 21 ± 1 kcal/mol. This value is identical to the value reported at pH 7.5 for HH16,

TABLE 3. Association and dissociation rate constants of I/II format hammerheads.

Hammerhead	k_{-1} (min^{-1})	k_1 (μM^{-1} min^{-1})	K_d (nM)[a]
HHα5	0.035 ± 0.015	2.2 ± 1.1[b]	16 ± 10
HHα1	0.4 ± 0.2	2.4 ± 0.5	166 ± 71

[a] Calculated from k_1 and k_{-1} values.
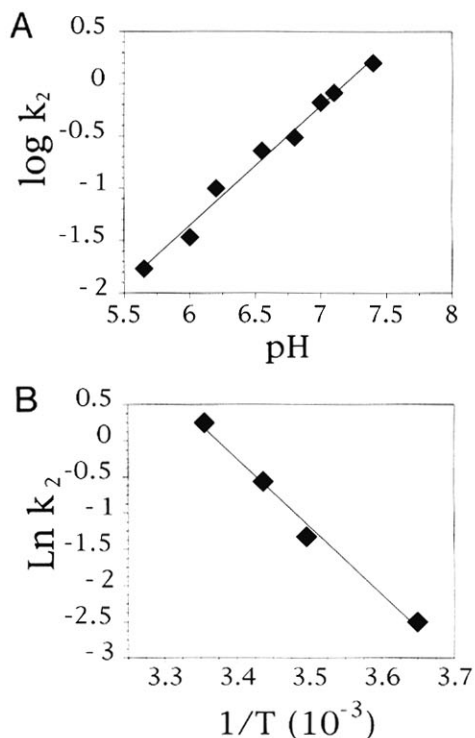[b] Calculated from k_{-1} and K_M measurements.

A

B

FIGURE 6. A: Rate of cleavage of HHα1 versus pH at 4 °C. The best fit line has a slope of 1.1. **B:** Arrhenius plot of the temperature dependence of the HHα1 rate constant of cleavage k_2 at pH 6.5, Ea = 21 ± 1 kcal/mol.

FIGURE 7. Rate of cleavage (k_2) of HHα1 versus magnesium at pH 6 fit to a hyperbolic binding equation giving an apparent K_{Mg} = 190 ± 30 mM.

a well-characterized I/III hammerhead (Hertel & Uhlenbeck, 1995).

The metal ion dependence of the cleavage rate for both HHα1 and HHα5 was investigated. The rate of cleavage was measured as function of magnesium concentration ranging from 1 mM to 1 M at a ribozyme concentration of 1 μM and trace substrate at pH 6 for HHα1 (Fig. 7), and at a ribozyme concentration of 10 μM and trace substrate at pH 6.5 for HHα5 (data not shown). The data fit well to a simple binding isotherm giving an apparent affinity constant, K_{Mg}, for the HHα1–Mg^{2+} complex of 190 ± 30 mM and for the HHα5–Mg^{2+} complex of 100 ± 30 mM. These values are at least five times higher than the ones reported for I/III hammerheads (Dahm & Uhlenbeck, 1991; Perreault et al., 1991; Yang et al., 1992; Dahm et al., 1993; Clouet-d'Orval et al., 1995). Cleavage rates of HHα1 were also determined in the presence of 10 mM Mn^{2+}, 10 mM Ca^{2+} (Dahm et al., 1993). Reactions were carried out at pH 6 to avoid insolubility problems caused by metal hydroxide (Dahm et al., 1993) with 1 μM ribozyme and trace substrate. The cleavage rate increases sixfold with manganese (k_2 = 3.5 min^{-1}) and decreases about 30-fold with calcium (k_2 = 0.018 min^{-1}) when compared with the rate of cleavage in 10 mM Mg^{2+} at pH 6 (k_2 = 0.57 min^{-1}). In the presence of 2 mM Co^{2+} and 2 mM spermine, the rate of
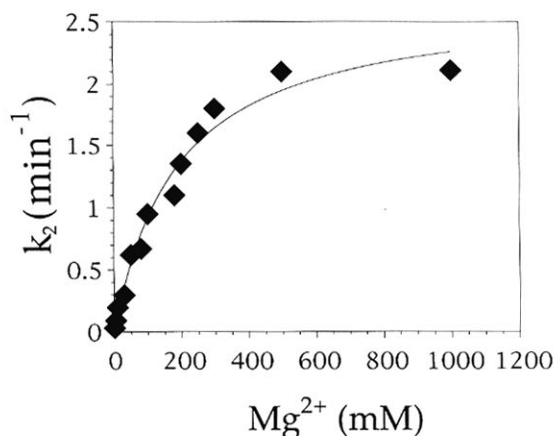
cleavage is 20-fold faster (k_2 = 0.38 min^{-1}) than the rate obtained with Mg^{2+} under the same conditions (k_2 = 0.018 min^{-1}). The effects of these metal ions on k_2 give a trend similar to that observed for a I/III hammerhead (Dahm et al., 1993).

DISCUSSION

Although ultimately successful, identifying a format I/II hammerhead where the ribozyme and substrate oligonucleotides did not form stable alternative conformers was more difficult than anticipated. Five different combinations of helix I and helix II sequences were chosen based on their similarity to well-behaved I/III format hammerheads. Despite the fact that none of the oligonucleotides were predicted to form extensive secondary structures, three of the ribozyme oligonucleotides formed multiple bands on nondenaturing gels. The different species are primarily intermolecular aggregates, but alternative intramolecular forms may also be present. Because the different species separate during electrophoresis, their rate of interconversion is expected to be slow. Thus, the three ribozymes that show multiple species on native gels are likely to show kinetic inhomogeneity and lower activity than a ribozyme that does not have stable alternative forms. For this reason, the kinetic characterization of these three hammerheads was not pursued.

The best previously characterized I/II hammerhead, O1/O2, provided an interesting control for the nondenaturing gel experiments. Previous melting experiments had shown that both oligonucleotides contained extensive secondary structure and an intramolecular hairpin had been proposed for O2 (Ruffner et al., 1989). When analyzed on nondenaturing gels, both O1 and O2 form multiple species in the concentration range typically used for kinetic experiments. Despite

apparent homogeneity in kinetic progress curves (Uhlenbeck, 1987), the presence of multiple stable forms of ribozyme and substrate oligonucleotides makes the O1/O2 hammerhead unsuitable for careful study. Indeed the structural heterogeneity is probably responsible for the fact that O1/O2 shows a relatively slow k_{cat} and an abnormal pH–rate profile when compared to other hammerheads (Uhlenbeck, 1987). The multiple forms of O1/O2 also make this hammerhead a poor choice for structure–function studies involving mutant or chemically modified hammerheads. A change in one of the nucleotides could alter the relative proportion of structural isomers and therefore alter the cleavage rate for a reason unrelated to the hammerhead reaction. Thus, the extensive structure–function data available using this hammerhead (Ruffner et al., 1990; Fu & McLaughlin, 1992a, 1992b; Fu et al., 1993) must be re-evaluated using a kinetically well-behaved system. Results of structure–function studies using other poorly characterized format I/II hammerheads (Koizumi & Ohtsuka, 1991) should also be interpreted with care.

The remaining two I/II hammerheads with no stable alternate conformers were studied in further detail. Attention was focused primarily on the association and dissociation constants between substrate and ribozyme, k_1 and k_{-1}, and the rate of cleavage, k_2. The rates of product dissociation were not studied extensively except to show that in a multiple turnover reaction, product release was rate limiting for HHα5 whereas it was not for HHα1. The comparatively weak binding of products prevented a study of the rate of the reverse reaction, k_{-2}.

The association rate constants obtained for the two I/II hammerheads were both approximately 2 μM^{-1} min^{-1}. This value is slower than the k_1 values from 9 to 100 μM^{-1} min^{-1} reported for several I/III hammerheads, including at least two with similar helix length and composition (Fedor & Uhlenbeck, 1992; Hertel et al., 1994). Hammerhead association rate constants are expected to reflect the second-order rate constant for RNA duplex formation, which has been measured for a number of short perfect duplexes to range between 30 and 720 μM^{-1} min^{-1} (Turner et al., 1990). Because this rate constant reflects helix nucleation, it is expected to vary only modestly with length and composition as long as G-C pairs are present, and it is not expected to be influenced by the hammerhead format. Two hypotheses could account for the unusually slow k_1 values for the I/II hammerheads. One possibility is that either the ribozyme or the substrate adopts an alternative conformation that greatly reduces the number of nucleation sites. However, unlike other hammerheads with slow k_1 values (Fedor & Uhlenbeck, 1992), no evidence for alternative structures on native gels was observed. A second possible explanation for the slow k_1 values is that the tertiary interactions in the core form sufficiently slowly that the assembly in-

termediate containing a single helix accumulates and frequently dissociates before the hammerhead can cleave. If the model is correct, the measured value of k_1 is reduced by an amount that depends upon the relative rates of intermediate dissociation and hammerhead core formation. Because the dissociation rates of helix I and helix II for HHα1 can be calculated from helix stabilities to be 6×10^4 min^{-1} and 9×10^3 min^{-1}, the rate of assembly of the hammerhead core would have to be about 200 min^{-1} to account for the observed k_1. Although 200 min^{-1} is much slower than rates for RNA helix elongation or hairpin loop formation, it is in the same range as that reported for tRNA tertiary structure formation (Crothers et al., 1974). More complex RNAs fold more slowly (Zarrinkar & Williamson, 1994). Other experiments will be required to investigate the origins of the slow k_1 values in these format I/II hammerheads.

In either hammerhead format, the rate of dissociation of substrate from ribozyme, k_{-1}, is expected to be strongly influenced by the stability of the two intermolecular RNA helices that join the two molecules. In the case of two different hammerheads in the I/III format, the value of k_{-1} was found to be 200–500-fold faster than the dissociation rate calculated for the corresponding continuous RNA helix with a sequence of helix I plus helix III (Hertel et al., 1994). This faster k_{-1} of the I/III hammerheads can be attributed to the destabilizing effect of the highly asymmetrical internal loop consisting of the cleavage site nucleotide on one strand and the remainder of the core on the other strand. In the case of the two I/II hammerheads studied here, a similar correlation between k_{-1} and RNA helix stability is expected. Indeed, the k_{-1} for HHα5 is substantially slower than that of HHα1, consistent with its more stable stem I. If one uses the free energy parameters for RNA helix formation (Freier et al., 1986) and assumes a $k_1 = 10^8$ M^{-1} min^{-1}, the k_{-1} values for the continuous RNA helices with sequences of helix I plus helix II are calculated to be 13- and 40-fold slower than the corresponding measured rates of HHα1 and HHα5. Thus, with this limited data set, it appears that the more symmetric internal loop formed by the core in the I/II hammerheads destabilizes an RNA helix somewhat less than was observed for the I/III hammerheads. It is unclear why the hammerhead core contributes somewhat differently in the two formats. Symmetric internal loops are indeed believed to be less destabilizing than asymmetric internal loops (Freier et al., 1986). In addition, stabilizing tertiary interactions must form in the I/II format, whereas in the I/III format, they are already partially formed. It will be interesting to see whether the hammerhead format influences the amount that different mutations destabilize the hammerhead core.

The rates of cleavage, k_2, for the two I/II hammerheads differ substantially from each other. At 10 mM

MgCl$_2$ (pH 7.5) and 25 °C, HHα5 has a $k_2 = 2.5$ min^{-1}, which is similar to the 0.5–2 min^{-1} reported for seven different I/III hammerheads in the same conditions (Fedor & Uhlenbeck, 1990, 1992; Hertel et al., 1994). In contrast, HHα1 cleaves substantially faster. Although an accurate single turnover cleavage rate was difficult to obtain by manual methods at pH 7.5, a multiple turnover rate of 10 min^{-1} for HHα1 was obtained. Furthermore, like other hammerheads, HHα1 shows a linear relationship between log k_2 and pH, so a value of $k_2 = 9.5$ min^{-1} at pH 7.5 could be extrapolated from single turnover rate data at lower pH values. Thus, HHα1 shows a rate of chemical cleavage about 10-fold faster than every other hammerhead previously studied. It should be noted that although several reports of hammerhead cleavage rates in excess of 10 min^{-1} have appeared (Sawata et al., 1993; Chartrand et al., 1995; Shimayama et al., 1995), the measurements were all done at higher pH values, magnesium concentrations, or temperatures, where the hammerhead reaction rate is known to be faster. When the reported rates of the "faster" hammerheads are extrapolated back to the "standard" buffer conditions used here, their k_2 values are in the range of 1–2 min^{-1}, and thus are much slower than HHα1. Similarly, because HHα1 also cleaves faster as pH, magnesium concentration, and temperature are raised, its cleavage rate is expected to substantially exceed the reported "fast" rates of the other hammerheads. Thus, the k$_2$ of HHα1 is intrinsically faster.

To investigate what could account for the fast rate, the properties of the rate of cleavage of HHα1 were examined and compared to those reported for other hammerheads. The linear relationship between log k_2 and pH observed for HHα1 has been observed for two I/III hammerheads (Dahm & Uhlenbeck, 1991; Dahm et al., 1993). The temperature dependence of k_2 gives an activation energy that is identical to a I/III hammerhead (Hertel & Uhlenbeck, 1995). The effects of different metal ions on k_2 suggest that, as with other hammerheads, a metal bound hydroxide ion acts as a base in the HHα1 cleavage reaction (Dahm et al., 1993). The magnesium dependence of k_2 for HHα1 revealed that much higher magnesium concentrations were needed to reach saturation than had been reported for HH16, a I/III hammerhead (Clouet-d'Orval et al., 1995). However, because HHα5 and at least one other I/III format hammerhead (unpubl. results) also show higher saturation values, it seems that neither the fast cleavage nor the format correlated with the altered magnesium ion binding properties. Thus, in general, the extrinsic properties of HHα1 cleavage are very similar to those observed with other hammerheads. The fast rate of cleavage is presumably the result of the sequence of stem I, because the slower HHα5 is identical elsewhere. The elements in stem I responsible for the fast rate await mutagenesis experiments.

MATERIALS AND METHODS

RNA synthesis

Substrate RNAs Sα1, Sα2, Sα3, Sα4, and Sα5 were synthesized by in vitro transcription of a synthetic DNA template with T7 RNA polymerase and subsequently gel purified (Uhlenbeck, 1987; Fedor & Uhlenbeck, 1992). Ribozyme RNAs Eα1, Eα2, Eα3, Eα4, and Eα5 were chemically synthesized and gel purified (Fedor & Uhlenbeck, 1992). RNAs 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.

Native gel assays

To test the structural homogeneity of the synthetic and transcript RNA, a 40 × 22 × 0.1-cm polyacrylamide gel was cast using 15% acrylamide (acrylamide/bisacrylamide, 19:1) in 50 mM Tris-acetate (pH 7.5) and 10 mM magnesium acetate. Radiolabeled and nonradiolabeled RNAs were combined in 50 mM Mops (pH 7.5), heated to 95 °C for 2 min, allowed to cool to 25 °C over 5 min, and then incubated for 10 min. Sucrose was then added to a final concentration of 5%. After a 2-h pre-electrophoresis, 25-μL samples were loaded at room temperature and electrophoresis was carried out at 4 °C at 10 W for 14 h.

Kinetics

Single-turnover cleavage rates were measured with an excess of ribozyme and a trace amount of 5'-^{32}P end-labeled substrate. Sulfonate buffers used were Mes (pH 5.7–6–6.3), Pipes (pH 6.6–6.8–7), and Mops (pH 7.2–7.5). A typical reaction was carried out in 20 μL. Ribozyme and trace [5'-^{32}P] substrate were heated in 50 mM buffer at 95 °C for 2 min and slowly cooled to 25 °C for 5 min. For the zero time point, 1 μL of the mixture was removed before adding magnesium and mixed in 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 magnesium to a final concentration of 10 mM. At specific time intervals, 2-μL aliquots were removed and quenched in 10 μL of stop solution. Substrate and product 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). For the experiment determining the magnesium dependence of the cleavage rates, 2-μL aliquots were quenched in 60 μL of 80% formamide, 0.1 M EDTA. As a result, even reactions in 1 M MgCl$_2$ contained an excess of EDTA and are expected to stop rapidly. Control experiments showed that no cleavage occurred after the stop buffer was added.

The upper limit of the substrate dissociation rate constant was determined by pulse–chase experiments (Herschlag & Cech, 1990; Fedor & Uhlenbeck, 1992; Hertel et al., 1994). A 40-μL reaction mixture containing 2 μM of ribozyme with a trace amount of radiolabeled substrate in 50 mM buffer was prepared. Half of this mixture was initiated by adding MgCl$_2$ to a final concentration of 10 mM. After an initial binding of period t_1, a chase was done by diluting 1 μL of the mixture reaction in 200 μL of dilution buffer (50 mM buffer, 10 mM

MgCl$_2$). During the chase period, 2-μL aliquots were removed and quenched in 10 μL stop buffer. The other 20 μL of the mixture was used as a control treated as a regular single-turnover reaction. No significant cleavage of radiolabeled substrate was observed in experiments in which the 1/200 dilution was made prior to initiation ($t_1 = 0$), indicating that the chase condition used did not allow rebinding of radiolabeled substrate. This experiment was repeated with three different buffers: Mops (pH 7), Pipes (pH 6.5), and Mes (pH 6). Depending on the pH, different times, $t_1 = 5$ s, 7 s, or 1 min, were used respectively for pH 7, 6.5, and 6.

The rate constant for substrate binding was measured by using pulse–chase experiments (Hertel et al., 1994). Several concentrations of ribozyme ranging from 5 to 200 nM in reaction buffer (Pipes, pH 6.5) were combined with trace amounts of radiolabeled substrate. For each ribozyme concentration, several chase reactions by 1/200 dilution were initiated at various times, t_1 ranging from 30 to 300 s, effectively quenching the binding of labeled substrate to ribozyme. At 1 h, greater than six times the $t_{1/2}$ for the cleavage reaction, the amount of product was measured. Under these conditions, $k_{obs} = k_{on}[S] + k_{off}$ (as described in Herschlag & Cech, 1990).

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