2092–2096 Nucleic Acids Research, 1995, Vol. 23, No. 12

The effect of base mismatches in the substrate recognition helices of hammerhead ribozymes on binding and catalysis

Martina Werner+ and Olke C. Uhlenbeck*

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

Received April 25, 1995; Revised and Accepted April 28, 1995

ABSTRACT

The ability of the hammerhead ribozyme to distinguish between matched and mismatched substrates was evaluated using two kinetically defined ribozymes that differed in the length and sequence of the substrate recognition helices. A mismatch in the innermost base pair of helix I affected k_2, the chemical cleavage step, while more distal mismatches had no such effect. In contrast, mismatches in any of the four innermost base pairs of helix III affected k_2. Chase experiments indicated that mismatches also increased the rate of substrate dissociation by at least 20–100-fold, as expected from the stabilities of RNA helices.

INTRODUCTION

The hammerhead motif is found in several plant pathogenic viroids and virusoids where it is involved in RNA self cleavage in the course of genome replication (1–4). Hammerheads can be assembled from two different RNA strands, one acts as the ribozyme and the other as the substrate (5–8). The separation of ribozyme and substrate has two major advantages: first, it permits kinetic and mechanistic studies of the cleavage reaction, and secondly, it permits the design of hammerheads that can cleave cellular or viral RNA genes at defined positions (6). Such sequence specific ribozymes could potentially be utilized as therapeutics, similar to antisense oligonucleotides (9–11). For hammerheads to bind and cleave a specific mRNA in a large pool of intracellular RNA molecules, the specificity of the reaction is crucial. The ribozyme has to be able to discriminate between the correct substrate and RNA sequences differing from the target sequence in only one position. The simplest model predicts that, like antisense oligonucleotides, the specificity of ribozyme cleavage depends on the difference in the affinities of binding between matched and mismatched targets (12). The affinity can, therefore, be adjusted with the length and base composition of the substrate recognition helices. Whereas a minimum number of base pairs is required to ensure that the targeted sequence is unique, recognition helices that are too long will potentially reduce the specificity of cleavage by reducing the difference in binding affinity between matched and mismatched substrates. Herschlag (13) has pointed out that the specificity of ribozymes is also greatly affected by the cleavage rate. If the cleavage rate of both matched and mismatched substrate is much faster than the rate of substrate dissociation, both will be cleaved no matter how large the difference in binding affinity. Optimal specificity is only achieved if the cleavage rate is in the same range as the rate of substrate dissociation. In this paper we examine the effect of single mismatches in the hammerhead recognition helices on the kinetics and specificity of the cleavage reaction.

MATERIALS AND METHODS

RNA synthesis

Ribozyme RNAs were synthesized using T7 RNA polymerase transcription of partially duplex synthetic DNA templates (14). Substrate RNAs were synthesized chemically with an automated synthesizer (Applied Biosystems 394) using ribonucleotide phosphoramidites (Applied Biosystems, Glen Research or Milligen). After base deprotection with ethanolic ammonia according to the instructions of the suppliers, the solvent was evaporated and the residue dissolved in 1 M tetrabutylammoniumfluoride in tetrahydrofuran and left at room temperature in the dark for 24 h (15). The deprotected oligo was desalted by gel filtration on Sephadex G25 column (NAP 10, Pharmacia) equilibrated with 10 mM Tris–HCl, 1 mM EDTA, pH 7.5.

Both chemically synthesized and transcribed RNA molecules were purified by gel electrophoresis under denaturing conditions on 20% acrylamide gels containing 8 M urea. The bands were visualized by UV shadowing and cut out. Following elution by crushing the gel slices and soaking in 0.1 M sodium acetate pH 6.5, the RNA was purified on a DEAE ion exchange chromatography column and subsequently precipitated with ethanol. Substrate RNA was 5′-end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Radioactive substrates were gel purified, electroeluted into 3 M sodium acetate using an IBI analytical electroeluter, and ethanol precipitated twice. Oligonucleotide concentrations were determined by assuming an extinction coefficient of 6600 M^{-1} cm^{-1} per residue.

The integrity of each chemically synthesized RNA substrate was confirmed by subjecting a portion of each oligomer to complete digestion by a mixture of ribonucleases: A, T1 and T2. The resulting nucleoside 3′ monophosphates were 5′-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and subsequently separated by two-dimensional thin layer chromatography (16). Fully deprotected oligonucleotides yielded four radioactive spots

* To whom correspondence should be addressed

+Present address: Innovir Laboratories, 510 East 73rd Street, New York, NY 10021, USA

at the same locations as the four nucleoside 5′,3′ bisphosphate standards, whereas partially deprotected RNAs resulted in a more complex TLC pattern.

Cleavage reactions

Cleavage reactions were carried out in 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$ at 25°C, unless otherwise indicated. To disrupt aggregates of RNA molecules which might form during storage, all samples were heated to 95°C for 1 min in 50 mM Tris–HCl and then cooled to 25°C (17). Two different protocols were used to initiate the cleavage reaction. In one, ribozyme and substrate in a volume of 23 µl were heated and cooled together and the cleavage reaction was started by adding 2.6 µl of 100 mM $MgCl_2$. In the second, 14 µl of ribozyme solution and 10 µl of substrate were heated and cooled separately, $MgCl_2$ was added to each to a final concentration of 10 mM and the reaction was started by mixing the two solutions. Cleavage rates measured using either protocol were the same, within error of measurement. Reactions were conducted with an excess of ribozyme (0.5–5 µM) and trace amounts of radioactive labeled substrate. At each timepoint, a 2 µl aliquot was removed and the reaction quenched with 10 µl stop mix containing 8 M urea, 50 mM EDTA and 0.1% of the dyes bromophenol blue and xylene cyanol. Substrate and product were separated on denaturing 15% polyacrylamide gels and the amount of radioactivity in each band was quantitated using a Molecular Dynamics PhophorImager. Each cleavage reaction was carried out for ~10 times the half-life, to ensure completion of the reaction. The extent of cleavage was typically between 85 and 95%. The amount of product formed P/(P+S) was determined at each timepoint and normalized to the final extent of cleavage. Each cleavage rate was determined at least twice and varied by <2-fold.

Chase experiments

The rate of substrate dissociation was measured using two different chase protocols. For each protocol, a 30 µl reaction was heated, cooled and started by the addition of $MgCl_2$. For the non-radioactive chase protocol (18), 2 µl of a 600 µM solution of unlabeled substrate was added 25 s after the initiation of the reaction. Two µl aliquots were removed at intervals and combined with 10 µl of stop mix. An identical control reaction without chase was carried out in parallel. For the dilution chase protocol, the entire reaction was diluted with 15–30 ml of reaction buffer shortly after the initiaton of the reaction (25 s to 10 min depending on the cleavage rate). Alternatively, 2 µl of the reaction mixture was diluted into 1 ml of reaction buffer in a siliconized reaction tube. The results of both protocols were the same. At each timepoint, a 50 µl aliquot was removed and mixed with 100 µl stopmix. The volume of these samples was reduced in a speed vac to ~70 µl before loading them into the wells of a 2 mm thick denaturing gel. Because the amount of radioactivity in each band was very low, those gels were exposed for several days on the PhosphorImager screen. An identical control reaction without the dilution was performed in parallel. The amount of product formed was determined at each timepoint as described above.

RESULTS

The two hammerheads used in this study have been well characterized kinetically. HH 15 (18) has five and seven base pairs in recognition helices I and III and HH 16 (19) has eight base

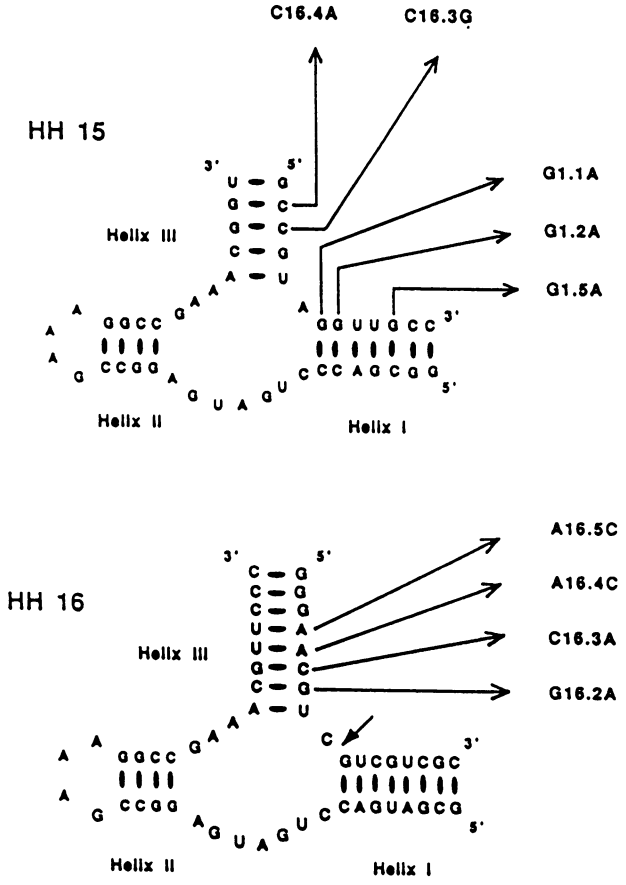

Figure 1. Sequence and secondary structure of HH 15 and HH 16. Both hammerheads consist of a ribozyme (R) and a substrate (S). The two parts of HH 15 are R15 and S15, HH 16 contains R 16 and S 16. Each arrow indicates the position of a single nucleotide mutation introduced in the substrate. The mutations are named according to the hammerhead nomenclature (20).

pairs in both helices. Under the conditions used in these experiments, substrates and ribozymes of both hammerheads do not aggregate or form alternate conformers to a significant extent. Single nucleotide mutations, named according to hammerhead nomenclature (20), were introduced in the substrates of both hammerheads as shown in Figure 1, resulting in mismatched base pairs at various positions in the recognition helices. With the exception of G16.2A mutation, all the mutations tested occur at positions where previous experiments (21,22) have indicated that base pair substitutions do not affect the cleavage rate. The recent X-ray crystal structure of the hammerhead (23) reveals that the complex central core of the hammerhead does not include any of the bases that were mutated, again with the exception of position 16.2. Thus, while all the mutations are expected to reduce the stability of the recognition helices and thus weaken substrate binding, only G16.2A might be expected to disrupt the catalytic core. While the consequences of the individual mismatches on helix structure and stability vary considerably, the focus of this study was to examine mismatches at various positions in the recognition helices to determine whether the general expectations are met. Future studies will focus on extensive examination of many mismatches at certain positions.

The cleavage rate k_2 was determined for each mismatched hammerhead using a trace concentration of radioactively labeled

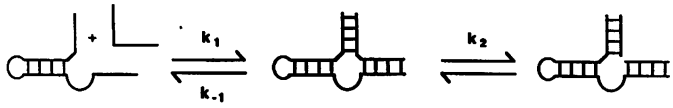

Figure 2. Schematic drawing of binding and subsequent cleavage of the substrate by the hammerhead ribozyme.

substrate and a saturating concentration of ribozyme (Fig. 2). Two experiments were performed to confirm that the observed rates did indeed reflect k_2. First, the cleavage rate was shown not to change over a 5–10-fold range of enzyme concentration, confirming that saturation was achieved. Secondly, each rate was shown to be 3–4-fold faster at pH 7.9 than at the standard pH 7.5. Since k_2 has been shown to be pH-dependent (24), this confirms that the observed rates reflect the cleavage step and not substrate binding or a conformational change.

The values of k_2 for the mismatched substrates are compared with the matched counterparts in Table 1. Mismatches in helix I were studied in HH 15. The mismatch closest to the core (G1.1A) reduced k_2 substantially, the mismatch at the next position (G1.2A) had a small effect, and the most distal mutation (G1.5A) gave the same k_2 as the fully matched substrate. Mismatches in the non-essential pairs in helix III were examined in the context of both HH 15 and HH 16 and the general pattern was similar for both hammerheads. For HH 16, k_2 for the innermost mutation (G16.2A) was 500-fold less than the matched substrate. Mutations at the next two positions (C16.3A, A16.4C) were ~10-fold less than the matched substrate and the most external mutation (A16.5C) has no effect on the cleavage rate. In HH 15, the k_2 of the 16.3 mismatch (C16.3G mutation) was nearly 600-fold less than for the matched substrate, and thus had a much larger effect than the corresponding mutation in HH 16. In contrast, the 16.4 mismatch in HH 15 has a modest effect that is quite similar to the corresponding HH 16 mutation.

While the effect of mismatches on k_2 had not been anticipated, mismatches are expected to increase the rate of substrate dissociation, k_{-1} since they destabilize the substrate helices. The extremely slow substrate dissociation of HH 16 (19) makes it inappropriate for these experiments. Instead, k_{-1} measurements were performed on the HH 15 mismatches. The value of k_{-1} for HH 15 had been originally determined by Fedor and Uhlenbeck (18), using a chase experiment. In this protocol, a trace of radioactive substrate was mixed with a saturating concentration of ribozyme and after a short period to allow annealing (10–20 s), a large excess of non-labeled substrate was added, and time points were taken to monitor cleavage of the radioactive substrate. A control reaction without the chase was run in parallel and k_{-1} was calculated from the ratio of the extent of cleavage at long times with and without chase ($F = k_2/(k_2 + k_{-1})$). When this protocol was repeated, a value of $k_{-1} = 1.6$ min^{-1} was obtained for HH 15 (Fig. 3A). While this value was in close agreement with the previous data (18), two lines of evidence suggested that this protocol does not give an accurate value of k_{-1} and the actual rate is substantially slower. First, a similar chase experiment performed with the G1.2A mismatched substrate gave a k_{-1} of nearly the same value as for the matched substrate. Since a C–A mismatch is expected to reduce the stability of an RNA helix by 2.3 kcal/mol (S. Freir, personal communication), the k_{-1} of a mismatched substrate was expected to be much faster than the matched. Secondly, the value of ΔG for substrate binding in HH 15 calculated from the experimental values of k_1 and k_{-1} (18) is anomalously weak when compared with the stability of the helical arms predicted from nearest neighbor values (25). For several other hammerheads, the ΔG of substrate binding was ~5 kcal/mol less stable than the calculated stability of the corresponding RNA helix, while HH 15 was nearly 10 kcal/mol less stable (19,26). Since the value of k_1 for HH 15 was similar to that found for other hammerheads, the anomalously weak substrate binding is potentially the result of an incorrect k_{-1} value. If HH 15 had a substrate binding affinity consistent with other hammerheads, its k_{-1} would be 10^{-4} min^{-1} or nearly 10 000-fold slower than was measured.

Table 1. Cleavage rates (k_2) for different substrates of HH 15 and HH 16

Substrate	k_2 (min^{-1})
S15	3.0 ± 0.3
S15 G1.1A	0.02 ± 0.002
S15 G1.2A	1.5 ± 0.2
S15 G1.5A	3.0 ± 0.3
S15 C16.3G	0.005 ± 0.0005
S15 C16.4A	0.4 ± 0.03
S16	1.8 ± 0.2
S16 G16.2A	0.003 ± 0.0005
S16 C16.3A	0.11 ± 0.03
S16 A16.4C	0.15 ± 0.02
S16 A16.5C	1.7 ± 0.2

These considerations prompted us to use a dilution chase protocol as an alternative approach to determine k_{-1}. In this protocol, the reaction is initiated as before, but after substrate binding occurs the reaction is diluted so that the concentrations of E and S are well below the K_d of the ES complex. Under these conditions, the rate of complex formation is so slow that no additional radioactive substrate can bind during the time of the reaction. As before, a difference will be seen in the extent of cleavage when the diluted reaction is compared to the undiluted control. When this protocol was used with HH 15, the control and the chase reaction were identical (Fig. 3B), indicating that all the bound substrate goes on to cleave and none dissociates during the time of cleavage. These data therefore indicate that k_{-1} must be ≥ 10-fold slower than k_2. In order to get a better estimate of k_{-1}, dilution chase experiments were conducted at both pH 6.1 and 5.5. At low pH, k_2 will be significantly reduced (24), thereby permitting determination of slower k_{-1} values. Under these conditions, a substantial fraction of the substrate dissociated in the course of the reaction, permitting an estimate of k_{-1} of 0.02 min^{-1} at both pH values (Fig. 3C).

The dilution chase protocol was then used to determine k_{-1} values for several of the mismatches in HH 15 (Table 2). For the G1.2A and G1.5A mutants, experiments performed at pH 7.5 did not give reliable data because k_2 was faster than substrate dissociation. However, at pH 7.0, values of 2 min^{-1} for G1.2A and 1 min^{-1} for G1.5A could be obtained. In contrast, the k_2 of the G1.1A mutation was so slow at pH 7.5 that all the substate dissociated before cleavage occured. However, when the chase experiment was performed at pH 8.8 where the value of k_2 is much faster, a value of $k_{-1} = 0.4$ min^{-1} was determined.

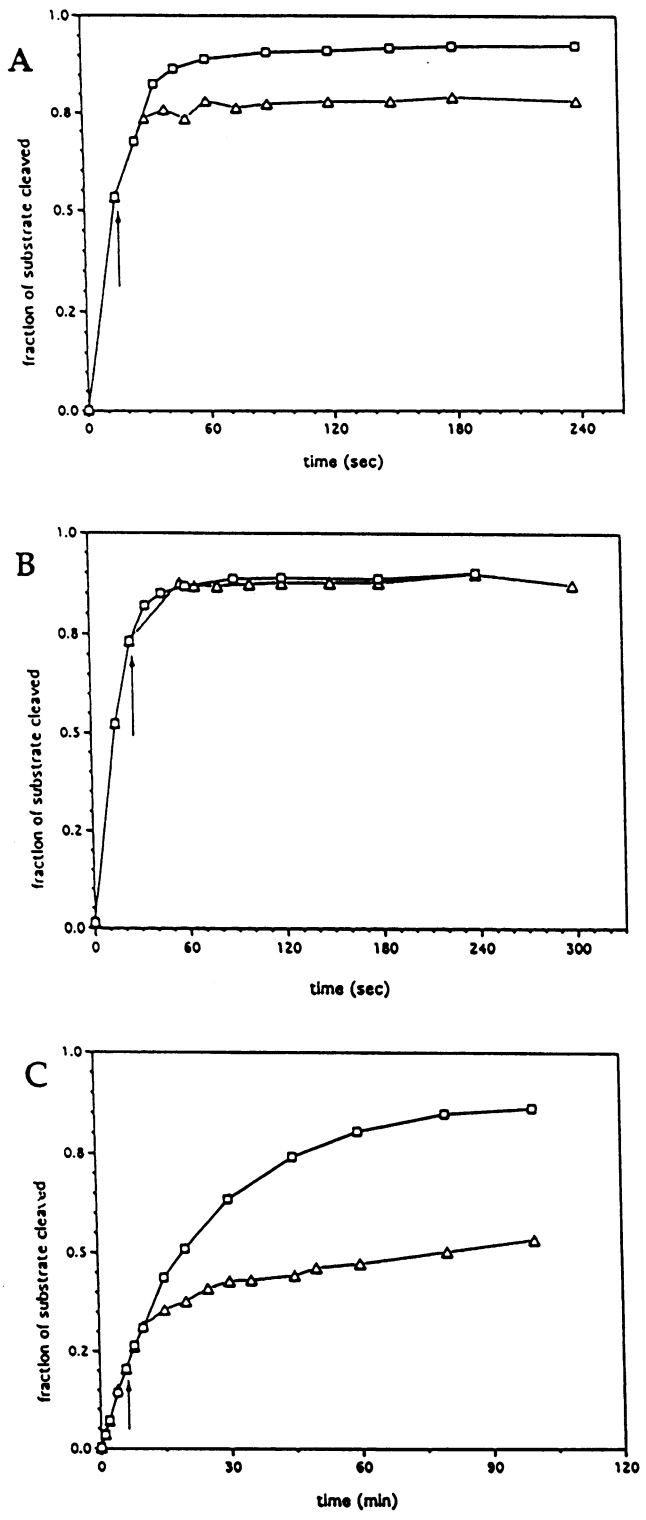

Figure 3. Chase experiments to determine substrate dissociation rate constant, k_{-1}. All experiments used 0.5 μM R15 and trace concentrations of [5′-^{32}P]S15. The control experiments are represented by open squares; the chase experiment by open triangles. (**A**) Chase experiment with non-radioactive substrates at pH 7.5. For the chase reaction, 40 μM S15 was added 15 s after initiation of the reaction (arrow). (**B**) Dilution chase experiment at pH 7.5. For the chase reaction, the reaction mix was diluted 1000-fold with pH 7.5 reaction buffer 25 s after the initiation of the reaction (arrow). (**C**) Dilution chase reaction at pH 6.1. For the chase reaction, the reaction mix was diluted 1000-fold with pH 6.1 reaction buffer 6 min after initiation of the reaction (arrow).

Table 2. Rates of substrate dissociation (k_{-1}) for different substrates of HH 15

Substrate	k_{-1} (min^{-1})	pH of measurement
S15	0.02 ± 0.01	6.1
S15 G1.1A	0.4 ± 0.3	8.8
S15 G1.2A	2 ± 1.5	7.0
S15 G1.5A	1 ± 0.5	7.0

DISCUSSION

Mismatches at certain positions in the substrate recognition helices reduce the rate of the catalytic step of the reaction. In the case of helix I, experiments with HH 15 indicate that this effect is restricted to the innermost base pair. The G1.1A mutation reduces the cleavage rate 150-fold while the more external mismatches do not affect cleavage significantly. Considering that residue 1.1 is adjacent to the cleavage site, this result is not surprising since it is likely that the 1.1–2.1 base pair is needed to maintain the position of the 5′ hydroxyl leaving group. Thus, when G1.1 is changed to an A, the position of the 5′ hydroxyl is either altered by the formation of an A–C pair or simply more mobile, resulting in a reduced cleavage rate. When mismatches are introduced further out in helix I, the structure of the G1.1–C2.1 pair is not affected and the cleavage rate is normal.

In contrast to the situation in helix I, nearly all the hammerheads with mismatches in helix III that were tested showed a reduced rate of the chemical step of the reaction. This is not surprising in the case of the G16.2A mutation in HH 16 since, as discussed above, the 15.2–16.2 base pair shows a clear sequence requirement (21) and the 2′-hydroxyl of position 15.2 makes a hydrogen bond with the 2′ OH of G5 in the catalytic core of the molecule (23). Thus, it would not be surprising if a C15.2–A16.2 mismatch would destabilize the core and reduce the cleavage rate. More surprising, however, is the observation that mismatches in positions 16.3 and 16.4 in both hammerheads showed reduced cleavage rates. Neither of these positions are involved in interaction with the catalytic core and some natural hammerheads appear to be fully active without the presence of a base pair at these positions. In the case of the HH 15, the mismatches may destabilize helix III sufficiently to denature it resulting in an inactive hammerhead which only contains helices I and II. The reduced value of k_2 would simply reflect the small proportion of molecules in which helix III was formed. While a similar explanation has been used to explain the reduced k_2 values of truncated substrates of HH 16 (26), it is less satisfactory as an explanation for the C16.3A and A16.4C mutations in HH 16. In this case, the mismatches are unlikely to be sufficient to fully denature helix III. It remains possible, of course, that the mismatches cause a local denaturation of helix III so as to disrupt the core. Mismatches in stem III of other hammerheads may help resolve this issue.

Our attempts to confirm the expectation that mismatches in the recognition helices will reduce the affinity of substrate binding were complicated by the fact that the rapid (1.6 min^{-1}) substrate dissociation rate, k_{-1}, previously deduced for HH 15 using a protocol involving a chase with non-radioactive substrate is likely to be incorrect. This conclusion was based on three observations. First, the experimental value of k_{-1} = 1.5 min^{-1} was much faster than a value of 10^{-4} min^{-1} predicted from a theory that accurately predicts the substrate binding affinity of other hammerheads (26).

Secondly, when an alternative dilution chase protocol was performed with HH 15, a much slower substrate dissociation rate was observed. Thirdly, the values of k_{-1} for the three mismatches in HH 15 determined here and a HH 15 truncated by two nucleotides (T. Stage, unpublished observation) are also in the order of 1 min^{-1}, a result inconsistent with the expectation that helix stability should affect dissociation rate.

It is unclear why the non-radioactive chase protocol gives an anomalously fast value of k_{-1} for HH 15. The protocol has given values of k_{-1} that are consistent with the dilution protocol in at least one other hammerhead. It is possible that the high concentration of the S15 in the chase inhibits the reaction in some way, although changing the concentration of S15 in the chase did not alter the results (18) and S15 in similar concentration did not affect the rate of HH 16 cleavage (M. Werner, unpublished observations). While it is possible that inactive complexes containing one ribozyme and two molecules of S could be forming in the chase experiment, no evidence for such trimeric complexes were observed on non-denaturing gels (18). In any case, it is clear that in order to obtain reliable kinetic data it is valuable to determine a rate constant using more than one protocol.

Since any chase protocol can only give an accurate value of k_{-1} if it is of the same order as k_2, the value of k_{-1} for HH 15 was too slow to be determined at pH 7.5. By performing the dilution chase experiment at pH 6.1, where k_2 is much slower, an estimate of $k_{-1} = 0.02$ min^{-1} was obtained. This value was confirmed in a separate experiment at pH 5.5 where k_2 is even slower. While these experiments give an estimate of k_{-1} that is considerably slower than the non-radioactive chase experiment, it must be taken with caution since it was measured at a fairly low pH. While no evidence for a pH dependence of k_{-1} was observed, we note that the experimental value is still 200-fold faster than the value predicted from helix stability.

When the dilution chase protocol was used for C~A mismatches in helix I, substrate dissociation rates between 0.4 and 2 min^{-1} were obtained. If we assume that dissociation rates are independent of pH, the mismatches increase dissociation rates by 20–100-fold. A C~A mismatch has been found to increase the ΔG of a RNA helix by 2.3 kcal/mol (S. Freier, personal communication) which corresponds to a 46-fold increase in the dissociation constant. Since single mismatches are not expected to greatly affect the association rate of RNA helices, the C~A mismatch would be expected to increase the dissociation rate 46-fold as well. Thus, quite good agreement between the experimental and calculated numbers is observed. Future experiments evaluating the effects of mismatches should use a hammerhead with a dissociation rate that is somewhat faster than HH 15, so that measurements of dissociation rates can be carried out in a narrower pH range. In any case, it is clear that, as expected, mismatches reduce substrate binding affinity substantially.

The results presented here indicate that hammerhead ribozymes are capable of distinguishing between matched and mismatched substrates in two different ways. First, as anticipated, mismatched substrates bind the ribozyme with a reduced affinity and thus will be cleaved at a lower rate under subsaturating conditions. The specificity of ribozyme cleavage will therefore depend on the length of the helices. Hammerhead ribozymes with short helices will have higher specificity, because the dissociation rate of mismatched substrates will be in the range of the cleavage rate. For hammerheads with longer helices such as HH 16, mismatched substrates will bind too tightly and will not cleave any differently than the matched substrate. Secondly, substrates with mismatches close to the hammerhead core have a reduced rate of cleavage and thus will be cleaved more slowly. This provides a second, unanticipated source of specificity that may be an advantage for using the hammerhead ribozyme instead of an antisense oligonucleotide in certain gene inactivation applications where discrimination between the matched and a known mismatched target is needed. In such an application, the target sequence must be appropriate such that the mismatch occurs within one or two residues of the required UpH dinucleotide sequence (H = U, C, A) 5′ to the cleavage site. If this can be arranged, one can expect that the mismatched target will cleave even slower than predicted by its reduced binding. Since an antisense oligonucleotide will primarily discriminate a mismatched target through reduced binding, the hammerhead should show better specificity. It would be valuable to test this prediction in a biological context.

ACKNOWLEDGEMENTS

This work was supported by a National Institutes of Health grant AI30242. We thank K. Hertel and T. Stage for helpful discussions.

REFERENCES

1 Prody, G.A., Bakos, J.T., Buzayan, J.M., Schneider, I.R. and Bruening, G. (1986) *Science* **231**, 1577–1580.
2 Buzayan, J.M., Gerlach, W.L. and Bruening, G. (1986) *Nature* **323**, 349–353.
3 Buzayan, J.M., Gerlach, W.L. and Bruening, G. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8859–8862.
4 Hutchins, C.J., Rathjen, P.D., Forster, A.C. and Symons, R.H. (1986) *Nucleic Acids Res.* **14**, 3627–3640.
5 Uhlenbeck, O.C. (1987) *Nature* **328**, 596–600.
6 Haseloff, J. and Gerlach, W. (1988) *Nature* **334**, 585–591.
7 Koizumi, M., Iwai, S. and Ohtsuka, E. (1988) *FEBS Lett.* **228**, 228–230.
8 Jeffries, A.C. and Symons, R.H. (1989) *Nucleic Acids Res.* **17**, 1371–1377.
9 Zamecnik, P.C. and Stephenson, M.L. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 280–284.
10 Ts'o, P.O., Miller, P.S. and Greene, J.J. (1983). In Cheng, Y.C., Goz, B. and Minkoff, M. (eds), *Development of Target Oriented Anticancer Drugs.* Raven Press, New York. p. 189.
11 Zamecnik, P.C. (1991). In Wickstrom, E. (ed.), *Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS.* Wiley-Liss, p. 1.
12 Freier, S.M. (1993). In Crooke, S.T. and Lebleu, B. (eds), *Antisense Research and Applications.* CRC Press, Inc., Boca Raton, Florida. pp. 67–82.
13 Herschlag, D. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 6921–6925.
14 Milligan, J.F., Groebe, D.R., Witherell, G.W. and Uhlenbeck, O.C. (1987) *Nucleic Acids Res.* **15**, 8783–8798.
15 Scaringe, S.A., Francklyn, C. and Usman, N. (1990) *Nucleic Acids Res.* **18**, 5433–5441.
16 Kuchino, Y., Watanabe, S., Harada, F. and Nishimura, S. (1980) *Biochemistry* **19**, 2085–2089.
17 Groebe, D.R. and Uhlenbeck, O.C. (1988) *Nucleic Acids Res.* **16**, 11725–11735.
18 Fedor, M.J. and Uhlenbeck, O.C. (1992) *Biochemistry* **31**, 12042–12054.
19 Hertel, K.J., Herschlag, D. and Uhlenbeck, O.C. (1994) *Biochemistry* **33**, 3374–3385.
20 Hertel, K.J., Pardi, A., Uhlenbeck, O.C., Koizumi, M., Ohtsuka, E., Uesugi, S., Cedergren, R., Eckstein, F., Gerlach, W.L., Hodgson, R. and Symons, R.H. (1992) *Nucleic Acids Res.* **20**, 3252.
21 Ruffner, D.E., Stormo, G.D. and Uhlenbeck, O.C. (1990) *Biochemistry* **29**, 10695–10702.
22 Ruffner, D.E., Dahm, S.C. and Uhlenbeck, O.C. (1989) *Gene* **82**, 31–41.
23 Pley, H.W., Flaherty, K.M. and McKay, D.B. (1994) *Nature* **372**, 68–74.
24 Dahm, S.C., Derrick, W.B. and Uhlenbeck, O.C. (1993) *Biochemistry* **32**, 13040–13045.
25 Freier, S.M., Kierzek, R., Jaeger, J.A., Sugimoto, N., Caruthers, M.H., Neilson, T. and Turner, D.H. (1986) *Proc. Nat. Acad. Sci. USA* **83**, 9373–9377.
26 Hertel, K.J. (1993) Ph.D. Thesis, University of Colorado.