Nucleic Acids Research, 1996, Vol. 24, No. 22 **4401–4406**

In vitro selection of hammerhead ribozymes containing a bulged nucleotide in stem II

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Received August 27, 1996; Revised and Accepted October 7, 1996

ABSTRACT

Hammerhead ribozymes were transcribed from a dsDNA template containing four random nucleotides between stems II and III, which replace the naturally occurring GAA nucleotides. *In vitro* selection was used to select hammerhead ribozymes capable of *in cis* cleavage using denaturing polyacrylamide gels for the isolation of cleaving sequences. Self-cleaving ribozymes were cloned after the first and second rounds of selection, sequenced and characterised. Only sequences containing 5′-HGAA-3′, where H is A, C or U, between stems II and III were active; G was clearly not tolerated at this position. Thus, only three sequences out of the starting pool of 256 (4^4) were active. The Michaelis–Menten parameters were determined for the *in trans* cleaving versions of these ribozymes and indicate that selected ribozymes are less efficient than the native sequence. We propose that the selected ribozymes accommodate the extra nucleotide as a bulge in stem II.

INTRODUCTION

The hammerhead ribozyme is an RNA motif which is capable of sustaining either *in trans* or *in cis* cleavage of a phosphodiester band (1–3) [for recent reviews see (4,5)]. The two-dimensional representation of the hammerhead ribozyme is depicted in Figure 1. Cleavage specificity is controlled by the hybridising arms of the ribozyme, which anneal with the substrate in a complementary fashion and direct cleavage of the scissile phosphodiester bond. This activity is specifically directed to occur after the third nucleotide of the cleavage triplet, at position H^{17}, for which cleavage is limited to sequences of the form 5′-NUH-3′ (where N is any nucleotide and H = A, U or C) (6–8). The ribozyme is composed of three α-helical regions, helices I, II and III, which flank the 11 single-stranded, conserved nucleotides of the catalytic core region. This sequence of conserved nucleotides has a particular tertiary structure, which has been elucidated by X-ray crystallography (9,10) and is further supported by a number of other biophysical techniques (11–13). As derived from the crystal structures, the catalytic core is built up of a base mispairing and a uridine turn domain (Fig. 1). Clearly, many of these tertiary interactions established from the crystal structures form the ground-state of the hammerhead ribozyme, with additional information on hydrogen bonding having been derived from chemical modification experiments (14–17). However, despite this wealth of data there is no clear indication of how catalysis is achieved by the hammerhead ribozyme.

The technique of *in vitro* selection (18–21) is of great interest in application to the hammerhead ribozyme since it would offer an opportunity to expand the number of nucleotide sequences after which cleavage can occur and would also provide information regarding the tertiary interactions within the conserved central core. Two *in vitro* selection strategies have already been applied to the hammerhead ribozyme and have confirmed the importance of forming a stable stem II structure for stabilisation of the adjacent $A^9{\cdot}G^{12}$, $G^8{\cdot}A^{13}$ double mismatches of the central core region (22,23). This was consistent with previous work by Tuschl and Eckstein (24), which highlighted the importance for a stem II of at least two G·C base pairs in order to attain optimal cleavage efficiency.

It was of interest to see if an extra nucleotide could be accommodated between stems II and III, as it can be between stems I and II (25), and whether there were rigid sequence preferences for this region. Thus, this could yield information regarding the conservation of nucleotides in this central core region, which form the mispairing domain observed in the crystal structures. A pool of ribozymes, containing four random nucleotides to replace G^{12}, A^{13} and A^{14}, was prepared by transcription from a DNA template. The active ribozymes were subsequently selected, as outlined in Figure 2, using a similar strategy as described by Nakamaye and Eckstein (23).

MATERIALS AND METHODS

Nucleoside triphosphates and 2′-deoxynucleoside triphosphates were purchased from Boehringer Mannheim. [α^{32}P]ATP (3000 Ci/mmol), [γ^{32}P]ATP (5000 Ci/mmol), [α^{35}S]dATP (3000 Ci/mmol), Sequenase quick-denaturing plasmid sequencing kit, *Taq* DNA polymerase and 10× reaction buffer, T4 polynucleotide kinase and 10× reaction buffer, MMLV-reverse transcriptase and 5× first strand buffer and Sequenase DNA polymerase were purchased from Amersham. X-Ray film (X-OMAT XAR-5) was purchased from Kodak. Radioanalytical scanning was performed on a Fuji BAS2000 Bio-imaging analyzer. *Eco*RI and *Bam*HI restriction endonucleases were purchased from NEB. Plasmid DNA purification columns and PCR QiaQuick spin columns were purchased from Diagen (Düsseldorf, Germany).

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Synthesis of oligonucleotides

The following DNA templates and primers were synthesised on an Applied Biosystems 380A DNA synthesiser and were purified as previously described (26): template-A (71mer) 5′-d(GCCA-CACTGA CTATAGTTCC CTATAGTXXX XGCTTGCGCT CATCAGAGTG TGGCTATAGT GAGTCGTTAT A)-3′, which contains the T7 promoter region (underlined); PCR-A (37mer), 5′-d(GCGCTA**GAAT TC**TATAACGA CTCACTATAG CCA-CACT)-3′, which restores the *Eco*RI restriction site (bold) and the T7 promoter region (underlined); RT-A (32mer), 5′-d(GGCGAT-**GGAT CC**GCCACACT GACTATAGTT CC)-3′, which restores the 3′ region, helix I, which is removed during the cleavage reaction and the *Bam*HI restriction site (bold).

Sequenase DNA polymerisation

Double-stranded DNA (pool 0) was prepared by mixing template A (40 µM, 10 µl) with PCR-A (40 µM, 10 µl). The strands were annealed by heating the solution to 70°C for 5 min and cooling to room temperature. The following reagents were then added to give the final concentrations indicated: Tris–HCl (pH 8.0, 65 mM), NaCl (50 mM), $MgCl_2$ (5 mM), dithiothreitol (5 mM), 2′-deoxynucleoside 5′-triphosphates (375 µM each). Sequenase DNA polymerase (1.3 U) was then added and the solution (50 µl) incubated at 37°C for 1 h. The dsDNA was recovered by ethanol precipitation using NH_4OAc (27).

T7 RNA transcription and RNA purification

dsDNA, produced from either Sequenase DNA polymerisation (pool 0) or from PCR (pool 1), was used as the template for the T7 RNA polymerase catalysed transcription. Each transcription was carried out in a volume of 250 µl with the following final concentration of reagents: DNA (1 µM), Tris–HCl (pH 8, 40 mM), $MgCl_2$ (20 mM), spermidine (1 mM), Triton X-100 (0.01%), dithiothreitol (10 mM), nucleoside 5′-triphosphates (4 mM each), T7 RNA polymerase (50 U/µl) and [α-^{32}P]ATP (in trace amount). Transcription mixtures were incubated at 37°C for 16 h (pool 0) or 8 h (pool 1), centrifuged and the supernatant removed from the pyrophosphate precipitate. The RNA transcribed was recovered from the supernatant by ethanol precipitation with NaOAc (27). The resultant RNA pellet was dried to remove any residual ethanol, dissolved in water (25 µl) and loaded onto a 12% denaturing polyacrylamide gel (0.4 mm thick, 50 W, 1.5 h). The desired length of RNA (46mer) was excised from the gel, using xylene cyanol as a marker, and the RNA extracted by suspending the crushed gel slice in NaOAc (1 M, pH 5.6, 200 µl). After incubating for 2 h on ice and, with brief vortexing every 30 min, the supernatant was removed. The extraction was repeated a second time, the supernatants combined and the RNA precipitated by addition of 3 vol ethanol. The pellet was then washed twice with ethanol/water (7:3, 2 × 150 µl) and the pellet thoroughly dried before being dissolved in water (25 µl).

RT–PCR

Reverse transcription was carried out in a 30 µl reaction volume with the following final concentrations of reagents: RT-A (833 nM), Tris–HCl (pH 8.0, 20 mM), KCl (100 mM), $MgCl_2$ (3 mM), gelatine (200 µg/ml), dNTPs (666 nM each), RNase inhibitor (1 U/µl) and MMLV-RT (0.67 U/µl). The protocol

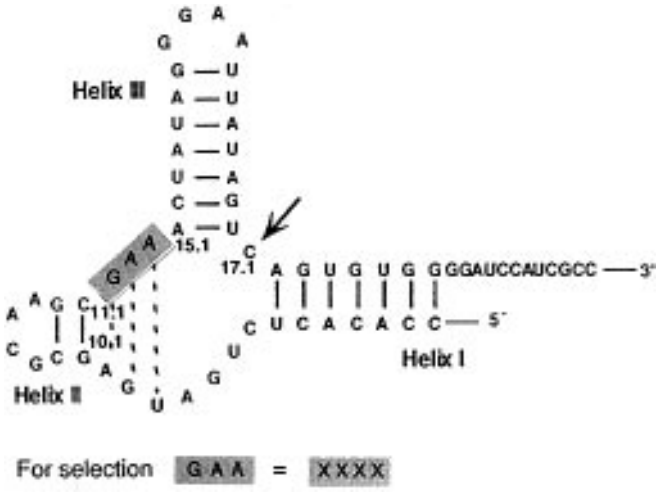

Figure 1. Sequence and secondary structure of the *in cis* cleaving hammerhead ribozyme used for the *in vitro* selection. Numbering is according to Hertel *et al.* (44); H: A, C or U; Bold lines indicate standard Watson–Crick base pairs; hatched lines depict mismatch base pairs predicted from the crystal structures (9,10).

observed was essentially that as described by Nakamaye and Eckstein (23) with the exception that after incubation for 1 h at 42°C the RNA in the mixture was destroyed by addition of NaOH (2 M, 20 µl) and water (150 µl). This was then incubated for a further 1 h at 37°C and the cDNA precipitated from this mixture by addition of NH_4OAc (10 M, 50 µl) and ethanol (750 µl). The cDNA pellet was then washed twice with ethanol/water (7:3) dried and dissolved in water (110 µl). Using the following PCR cycle, 94°C (30 s), 55°C (10 s), 72°C (2 min), as described by Long and Uhlenbeck (22), the number of PCR cycles required to amplify the cDNA was established by removing 10 µl aliquots every fifth cycle and determining the extent of product formation by agarose (2.5%) gel electrophoresis. The remainder of the cDNA was then amplified in ten 100 µl reactions. The DNA produced by this procedure was isolated by two ethanol precipitations; first of all using NH_4OAc, to remove the triphosphates, and then from NaOAc. This DNA was suitable for use in a further T7 transcription reaction or for cloning and sequencing. Cloning and sequencing was carried out as previously described by Nakamaye and Eckstein (23).

Kinetics of intermolecular ribozyme cleavage

The Michaelis–Menten parameters for *in trans* ribozyme cleavage were carried out under single turnover (28) and multiple turnover (24) conditions as previously described.

RESULTS

The selection experiments described here were carried out using a previously described procedure (23; Fig. 2), and the topic of *in vitro* selection has been extremely well reviewed (18–21). In brief, the protocol consisted of transcription of a random pool of RNA from a random DNA template and isolation of the shorter cleavage product (46mer) on a 12% denaturing polyacrylamide gel. The RNA was reverse-transcribed using primer RT-A, which restores the 3′-nucleotides lost through the cleavage and includes the *Bam*HI restriction site. The resultant cDNA was amplified by

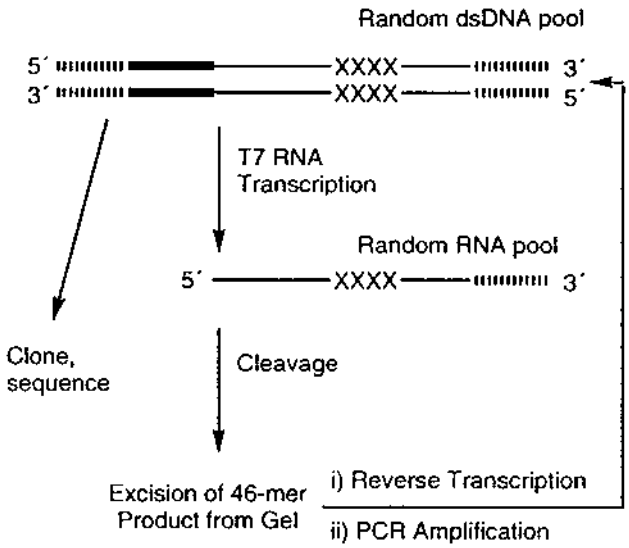

Figure 2. Selection cycle for isolation of cleaving hammerhead ribozymes. X, Random nucleotides; hatched line, 5′ *Eco*RI and 3′ *Bam*HI restriction sites; bold line, T7 promoter; line, constant transcribed nucleotide region.

PCR using, in addition to RT-A, the primer PCR-A, which restores the T7 RNA polymerase promoter and the *Eco*RI restriction site. The dsDNA was subsequently utilised for cloning or as a template in subsequent selection cycles.

During the selection the enrichment of each of the pools with *in cis* cleaving hammerhead ribozymes was established by transcribing each pool and measuring the extent of cleavage after 3 h incubation. RNA transcribed from pools 0, 1 and 2 DNA cleaved to approximately 4, 24 and 65% respectively and DNA pools 1 and 2 were cloned and sequenced to yield the following sequence data.

Pool 1 DNA

Forty-eight colonies were picked and from the subsequent run-off transcripts 10 of the clones appeared to give a cleavage product. These ten and a further eight clones, which demonstrated no cleavage, were sequenced and fell into the following four categories.

(i) Self cleaving ribozymes. Seven clones fell into this category and only three sequences were represented, where the random region was AGAA (3 times), CGAA (2 times) and UGAA (2 times). These three motifs cleaved to 82, 89 and 91% (Fig. 3) during a 30 min transcription reaction, which is similar to the 95% of the native hammerhead sequence.

(ii) Truncated sequences. Two sequences had lost nucleotides and gave rise to transcription products having the same length as the desired cleavage product. One had lost nine nucleotides from L2.3 to 15.2 and the other one eight nucleotides from 11.1 to 15.5.

(iii) Alternative cleavers. One sequence gave what appeared to be a cleavage product of ~10 nucleotides longer than desired. This had the random region as ACTT.

(iv) Non-cleavers. No similarity was present in the random region and the sequences found were AGTT, GGCA, CTCT, TATT, CATG, AGAT, GGTC and TTGA. Although these are termed non-cleaving ribozymes these sequences gave a background cleavage of ~5% (see GGCA, Fig. 3).

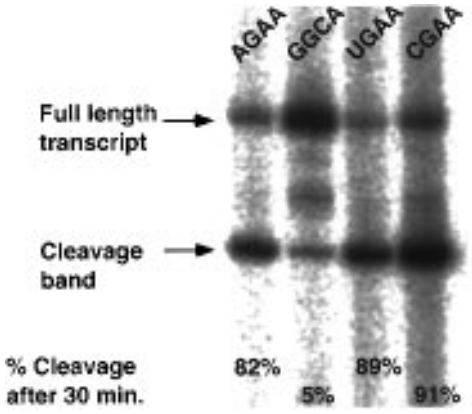

Figure 3. Percentage of *in cis* cleavage from T7 run-off transcriptions from three self-cleaving and one of the non-cleaving clones.

Pool 2 DNA

From the 15 clones picked and transcribed *in vitro*, nine supported *in cis* cleavage. Of these nine the sequences CGAA (7) and AGAA (2) were the only representatives.

Table 1. *In trans* cleavage with the 19mer substrate[a]

Ribozyme	k_{cat}' (min^{-1})	K_m' (nM)	k_{cat}'/K_m' ($\mu M^{-1}min^{-1}$)
GAA	0.84	127	6.6
CGAA	0.54	117	4.6
UGAA	0.37	143	2.6
AGAA	0.3	151	2

[a]Single turnover conditions using 25 nM substrate and ribozyme concentration ranging from 100 to 400 nM.

Table 2. *In trans* cleavage with the 12mer substrate[a]

Ribozyme	k_{cat} (min^{-1})	K_m (nM)	k_{cat}/K_m ($\mu M^{-1}min^{-1}$)
GAA[b]	4.7	140	33
CGAA	0.43	320	1.3
UGAA	0.38	150	2.5
AGAA	0.34	370	0.9
Rz A-bulge	0.34	414	0.8

[a]Multiple turnover conditions using 5–25 nM ribozyme and 50–1500 nM substrate concentrations.
[b]Taken from ref. 24.

In trans cleavage of selected ribozymes

Ribozyme sequences were synthesised for *in trans* cleavage and targeted against a 19mer substrate (Fig. 4, left; Table 1), which contains the sequences in helices I and III used in the selection experiment, and also against a 12mer substrate (Fig. 4, right; Table 2), for which the native ribozyme has been well characterised in this laboratory (24). Using the 19mer substrate (Fig. 4, left;

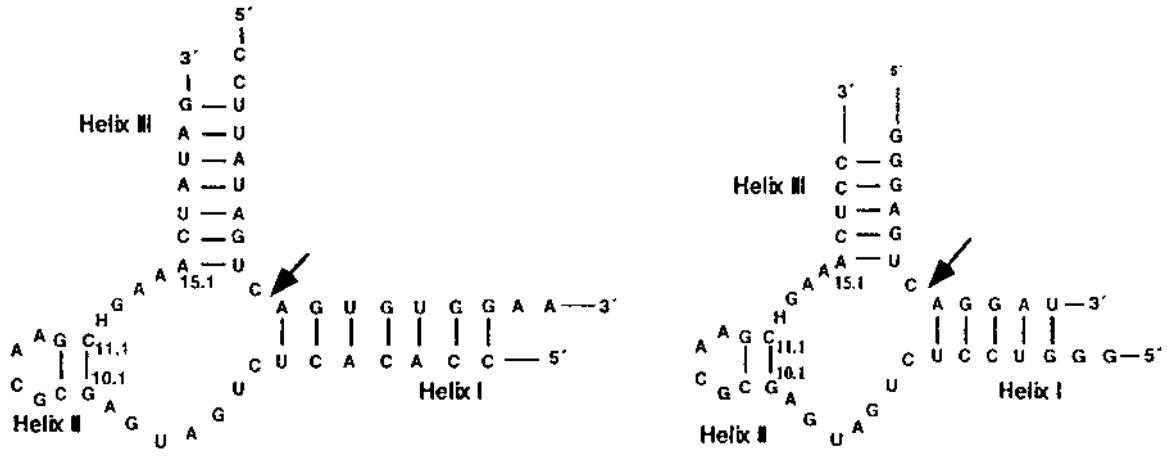

Figure 4. *In trans* cleaving ribozyme sequences. (Left) Ribozyme sequence directed against the 19mer substrate. (Right) Ribozyme sequence directed against the 12mer substrate

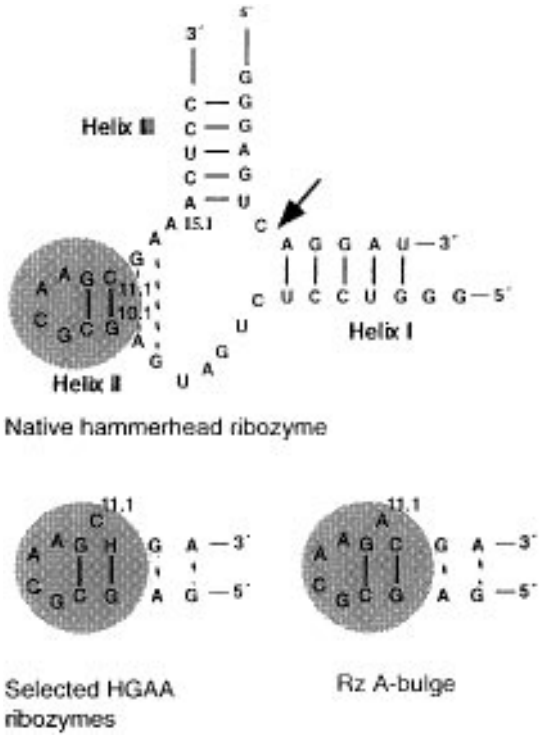

Figure 5. Secondary structures of hammerhead ribozymes. Bold lines indicate standard Watson–Crick base pairs; hatched lines depict mismatch base pairs predicted from the crystal structures (9,10); shaded region highlights the stem–loop II region; H: A, C or U.

Table 1) no cleavage was observed under multiple turnover conditions and Michaelis–Menten parameters were established using single turnover conditions. The three selected ribozymes had catalytic efficiencies only 3-fold lower than the native sequence.

Multiple turnover cleavage could be observed when the ribozymes were targeted against a 12mer substrate (Fig. 4, right). The results (Table 2) indicate that they cleave the substrate with between 10- and 37-fold lower catalytic efficiency, compared to the native GAA sequence. In order to investigate the possibility that the additional nucleotide is incorporated into stem II, forcing $C^{11.1}$ into bulge, Rz A-bulge was synthesised (Fig. 5), which contains an adenosine to replace $C^{11.1}$. This ribozyme was targeted against the 12mer substrate (Table 2) and demonstrated a similar catalytic efficiency to the selected ribozymes.

DISCUSSION

In vitro selection has already been utilised in hammerhead ribozymes in order to examine the importance of the stem–loop II region in the cleavage reaction (22,23). This confirmed the importance of a $G^{10.1}\cdot C^{11.1}$ base pair for the closing of stem II and the base mismatch region, formed by the $A^{9}\cdot G^{12}$, $G^{8}\cdot A^{13}$ and $U^{7}\cdot A^{14}$ mispairs, which connects stems II and III in a near continual α-helix (Fig. 1). The activity of hammerhead ribozymes containing shortened stem II sequences (24) have demonstrated that $G^{10.1}\cdot C^{11.1}$ and $C^{10.2}\cdot G^{11.2}$ base pairs are the minimum requirement for optimal catalytic efficiency. Ribozymes with a four nucleotide linker between $G^{10.1}$ and $C^{11.1}$ are also functional but cleave with less than one tenth the activity of ribozymes containing a stem II (24,29). *In vitro* selection also identified a ribozyme similar to the hammerhead as one of the self-cleaving motifs, amongst a variety of others, isolated from a pool of tRNA molecules containing a 100 nucleotide random insert in one of the loops (30).

In this paper a part of the central core region has been randomised to extend this selection technique to investigate the nucleotide requirements of the single-stranded region between nucleotides 11.1 and 15.1. The extra nucleotide was added, since it has been reported that an additional nucleotide can be incorporated into the single-strand region between stems I and II (31) without significant loss of ribozyme activity.

Selected sequences

Enrichment of the random pools with cleaving sequences was achieved with each round of selection and the DNA from pools 1 and 2 were cloned and sequenced to show how the selection progressed as the incubation time was lowered from 16 to 8 h. Pool 1 had three sequences with self-cleaving activity; *viz.* AGAA, represented three times and CGAA and UGAA, each

represented twice. The DNA from pool 2 had only two cleaving sequences; *viz.* CGAA seven times and AGAA twice, UGAA was not present. It is not clear why this selection favours the CGAA sequence so strongly, since from the run-off transcripts of the cleaving clones (Fig. 3) all three selected sequences cleave to ~85–90%. It is very probable that UGAA is in fact represented in pool 2, since only 15 colonies were picked for analysis and it may have been detected if a larger number of colonies had been screened.

All the selected ribozymes were synthesised chemically and their *in trans* cleaving parameters established (Tables 1 and 2). Using the 19mer substrate (Fig. 4a, Table 1), which contains the hybridising arms used in the selection cycle, the selected ribozymes displayed catalytic efficiencies similar to that of the native. In contrast, when the 12mer substrate was used (Fig. 4b, Table 2), the selected ribozymes had catalytic efficiencies which were up to 37-fold lower than the native.

The selected ribozymes, whether targeted against the 19- or 12mer substrate, all have similar catalytic efficiencies. With the 19mer substrate, efficiencies were only slightly lower than the native and this was mainly due to a slight decrease in $k_{cat'}$. That $k_{cat'}$ for the native is so much lower than k_{cat} obtained with the short substrate under multiple turnover could be due to the existence of a preequilibrium for the long ribozyme–substrate complex (32–34). With the 12mer substrate, the catalytic efficiencies of the selected ribozymes were lower than the native by over a factor of ten. The catalytic efficiency of this ribozyme has been shown to be very dependent upon the structure of stem–loop II, with variations in the loop sequence lowering the catalytic efficiency by up to 3-fold (24). Presumably the helical destabilisation caused by the additional nucleotide lowers the catalytic efficiency of the selected ribozymes.

The much lower catalytic efficiencies could also be a consequence of the selection having been carried out using different hybridising arms, although it has never been demonstrated that the sequence of stems I and III affect the efficiency of ribozyme cleavage. The trend in catalytic efficiency is reflected mainly by a lowering of the $k_{cat'}$ or k_{cat} values, whilst $K_{m'}$ or K_m remains relatively constant. Thus, the selected ribozymes appear to bind the substrate equally as well as the native hammerhead. Only with the 12mer substrate did CGAA, AGAA and Rz A-bulge demonstrate an ~2- to 3-fold increase in K_m, which is indicative of the presence of alternative conformations inhibiting substrate binding (35).

Structure of the selected ribozymes

The selected ribozymes indicate that 5′-HGAA-3′ is the only sequence tolerated in an active hammerhead ribozyme. The additional nucleotide was only observed at the 5′-end of the sequence, suggesting that it is incorporated into stem II and cannot be tolerated in the central core or stem III. This is reasonable since nucleotides 15.1 and 15.2 base pair with conserved nucleotides 16.1 and 16.2, which are the first two nucleotides of the cleavage triplet. As a result, there is no real possibility for accommodation of an additional base at the bottom of stem III. It is sensible therefore that the additional nucleotide is accommodated into stem II, where there is greater sequence tolerance for forming the closing base pair, thus forcing $C^{11.1}$ into a bulged position (Fig. 5). Although in most naturally occurring hammerhead ribozymes a $G^{10.1}{\cdot}C^{11.1}$ base pair is found, a $C^{10.1}{\cdot}A^{11.1}$ mismatch is present in the small barley yellow dwarf virus (sBYDV) (36) and an additional uridine is found between nucleotides A^9 and $G^{10.1}$ in the (+) strand of the lucerne transient streak virus (25). However, *in vitro* selection on the sBYDV sequence demonstrated that although a mismatch in stem II was tolerated, it was not optimal for the cleavage reaction and randomisation of positions 7, 10.1 and 11.1 yielded the more active sequence containing the standard $G^{10.1}{\cdot}C^{11.1}$ base pair (23).

Rz A-bulge (Table 2, Fig. 5) was synthesised to test the idea that the selected ribozymes accommodated the additional nucleotide by placing $C^{11.1}$ into a bulged position in stem II and creating a base pair between $G^{10.1}$ and the first random position. If this was the case, then a ribozyme containing an A-bulge would be as active as the CGAA selected ribozyme, which contains a C-bulge. The Michaelis–Menten parameters of this sequence (Table 2), targeted against the 12mer substrate, are only slightly different from that of the CGAA sequence implying that an A-bulge is tolerated fairly well in stem II. Bulges are very common in RNA structures and helical destabilisation and/or disruption is dependent on the flanking base pairs. However, generally they create a kinking of the helix by around 10° (37,38) and are not found to be particularly destabilising. Therefore, it is not unreasonable that the additional nucleotide could be accommodated as a bulge in stem II, and this would presumably be less destabilising to the overall structure of the hammerhead ribozyme than if it needed to be incorporated between stem II and the G/A mismatches. The double G/A mismatch extends the α-helix of stem II into the central core and it is an essential structural feature of the hammerhead ribozyme. Double G/A mismatches are common in RNA structures and, depending on the closing base pairs, they do not significantly destabilise helical DNA (39,40) or RNA (41–43). The drop in catalytic efficiency from CGAA to UGAA and down to AGAA, in particular with the 19mer substrate, is consistent with the formation of a progressively less stable $G^{10.1}{\cdot}H$ base pair. The results imply that the non-Watson–Crick $G^{10.1}{\cdot}A$ and a $G^{10.1}{\cdot}U$ base pairs, formed by ribozymes AGAA and UGAA respectively (Fig. 5), do not impair formation of the double G·A mismatches.

A bulge can be accommodated into stem II where it is part of an extended α-helix with a distal double G·A mismatch. Thus, the lower catalytic efficiency of the selected ribozymes (Table 2) can be explained by the destabilisation of stem II through the introduction of a bulged nucleotide. This also offers an explanation for the absence of GGAA as a cleaving sequence since a G cannot form a stable mispair with $G^{10.1}$.

Conclusions

Hammerhead ribozymes were selected from a pool containing four random nucleotides incorporated between helices II and III. These experiments demonstrate that the GAA sequence between stems II and III is a very strongly conserved motif. The additional nucleotide is most likely incorporated into stem II causing nucleotide $C^{11.1}$ to bulge out. All the selected sequences cleave *in cis* and *in trans* cleavage efficiencies are only slightly lower than the native.

ACKNOWLEDGEMENTS

We are extremely grateful to P. A. Heaton, Y. Berlin and K. Birikh for the critical reading of the manuscript and U. Kutzke for expert technical support. We thank one of the referees for pointing out

the possible existence of a preequilibrium in one of the complexes. This work was supported by the Deutsche Forschungsgemeinschaft and an EMBO fellowship to S.Th.S.

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