Selection of Hammerhead Ribozyme Variants with Low Mg^{2+} Requirement: Importance of Stem-Loop II

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Variants of the hammerhead ribozyme with high in trans (intermolecular) cleavage activity at low Mg^{2+} concentrations were in vitro selected from a library with 18 nucleotides randomised in the core and in helix II. The most active hammerhead ribozyme selected had the same sequence as the consensus ribozyme in the core but only two base pairs in stem II, G(10.1) – C(11.1) and U(10.2) – A(11.2), and a tetrauridine loop II. This ribozyme (clone 34) was found to be very active in single-turnover reactions at 1 mM Mg^{2+} concentration in the context of several substrates with differences in the lengths of stem I and III, including the well-characterised HH16 substrate and a derivative thereof with a GUA triplet at the cleavage site, as well as a substrate used previously in a related study. For the HH16 substrate, a change of base pair 10.2 – 11.2 to C – G in stem II further improved activity by about 2.5-fold to 0.8 min^{-1} (at 1 mM Mg^{2+} concentration, 25 °C, pH 7.5). Interestingly, this very active variant was not identified by the selection procedure. Changing loop II from UUUU to GCAA or extension of stem II to three or four base pairs reduced the cleavage rate by 2.0 – 2.5-fold. Thus, small hammerhead ribozymes carrying a tetrauridine loop with two base pairs in stem II represent the most active versions known so far at low Mg^{2+} concentrations; single-turnover rates of approximately 1 min^{-1} are reached at 25 °C and pH 7.5 in monophasic reactions, with endpoints between 75 and 90 %. Such constructs promise to be advantageous for the inhibition of gene expression in vivo.

KEYWORDS:

hammerhead ribozyme · metalloenzymes · ribozymes · RNA structure

Introduction

The hammerhead ribozyme cleaves substrates in an Mg^{2+}-concentration-dependent manner.[1–3] Despite many efforts the precise role of the metal ion has not yet been firmly established. It is generally accepted that it is essential for achieving the catalytically competent conformation, as has been shown by several studies, particularly fluorescence studies.[3] However, whether the metal ion directly participates in catalysis as an acid – base, as previously suggested,[4] is still a matter of debate, particularly as monovalent cations such as Li^+ and NH_4^+ can also support activity at higher concentrations.[5, 6] The optimal concentration for activity is 10 mM Mg^{2+} ions although rates increase further with higher concentrations; saturation is approached at 50 mM. The requirement of 10 mM Mg^{2+} concentration is much higher than that of free Mg^{2+} ions in mammalian cells, which is approximately 500 μM.[7, 8] Ribozymes with a lower requirement for Mg^{2+} ions should therefore be advantageous for the inhibition of gene expression.[9–11]

Results and Discussion

In vitro selection

The aim of this project was to identify hammerhead ribozyme sequences which would be active at lower Mg^{2+} concentrations than the standard in vitro conditions of 10 mM. This was approached by in vitro selection utilising a double-stranded-DNA (dsDNA) pool with 18 nucleotides randomised in the core and stem-loop II. Pool DNAs also encoded the T7 promoter and restriction sites for cloning which, however, were not made use of in the end. The in trans (intermolecular) selection set-up followed a report by Ishizaka et al.[12] and has also been adopted by Conaty et al.[13] The substrate with a GUC triplet for cleavage was biotinylated for immobilisation on streptavidin beads (Figure 1). Transcripts of the dsDNA pool containing the ribozyme population were incubated with the immobilised substrate. Addition of Mg^{2+} ions resulted in substrate cleavage by the active ribozyme sequences. Ribo-

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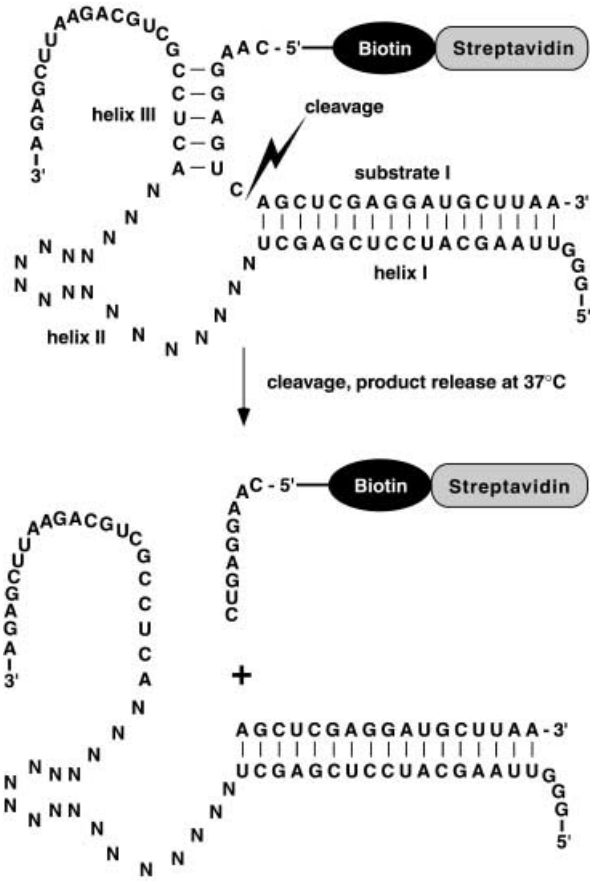
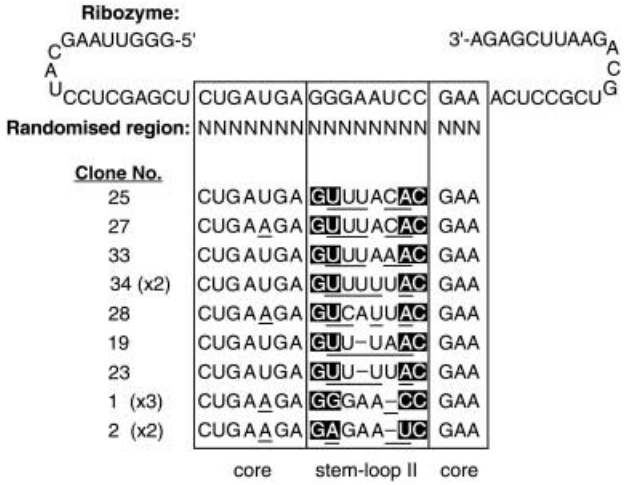


Figure 1. Ribozyme selection scheme. The substrate strand (substrate I), carrying a biotin moiety at the 5′-end, was immobilised on strepatavidin-coated beads. Hybrid helix III, which became the only attachment site for the ribozyme to the substrate strand after ribozyme cleavage, rapidly dissociated at 37 °C resulting in the release of active ribozymes from the support (for further details, see the Experimental Section). Residues of the randomised region are indicated as N.



Clone No.			
25	CUGAUGA	GUUUACAC	GAA
27	CUGAAGA	GUUUACAC	GAA
33	CUGAUGA	GUUUAAAC	GAA
34 (x2)	CUGAUGA	GUUUUUAC	GAA
28	CUGAAGA	GUCAUUAC	GAA
19	CUGAUGA	GUU–UAAC	GAA
23	CUGAUGA	GUU–UUAC	GAA
1 (x3)	CUGAAGA	GCGAA–CC	GAA
2 (x2)	CUGAAGA	GAGAA–UC	GAA

Figure 2. Clones of ribozymes selected for activity at low Mg^{2+} concentrations. Nucleotide identities deviating from the sequence of the native hammerhead ribozyme are underlined. Putative nucleotides forming stem II that encloses loop II are highlighted.

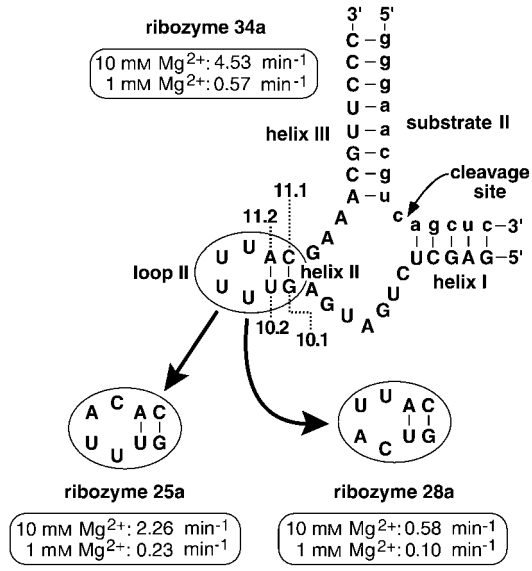


Figure 3. Sequences and single-turnover cleavage rates of three ribozymes obtained in the selection process (for further details, see the text and Table 1). Substrate II (in lower case letters) was utilised for the kinetic analyses, instead of the initial substrate I that was used for the selection procedure (Figure 1). Ribozymes 25a, 28a and 34a only differ from each other in the loop II sequence marked by open ovals; the numbering of stem II nucleotides is indicated.

zymes able to cleave the substrate were released at 37 °C to the supernatant, reverse transcribed and amplified by PCR for another cycle of selection with new substrate. Selection pressure was increased by lowering the Mg^{2+} concentration from 20 mM to 1 mM and the time of incubation from 8 minutes to 30 seconds.

After eight cycles of selection individual members of the complementary DNA (cDNA) pool were cloned. 48 clones (24 white and 24 blue) were chosen for sequencing, and 13 turned out to have a hammerhead-like core structure (Figure 2). 5 clones, deviating from the hammerhead design, were tested for activity and preliminary results show that they are inactive; they were therefore not further analysed in this study (data not shown). Clones with a conventional hammerhead core were chemically synthesised for kinetic analysis. For this purpose, the initial substrate I (Figure 1) was changed to a variant with nine nucleotides (nt) upstream and five nt downstream of the cleavage position, which results in a more common hybrid stem I length (substrate II, Figure 3). The ribozymes were changed accordingly (for example, derivative ribozymes Rz25a, Rz28a and Rz34a in Figure 3). Clone 25 was analysed as a representative of clones 25, 27 and 33, all of which were capable of forming a two-basepair (bp) minihelix II capped by a UUAC or UUAA tetraloop. For another subclass (clones 1, 2, 19 and 23) formation of a two-bp stem II and an unfavourable trinucleotide loop was predicted. Two representatives, clones 1 and 2, turned out to be only weakly active (>30-fold activity reduction compared with clone 34). Thus, clones 19 and 23 were not further analysed. The single-turnover rates measured at 1 and 10 mM Mg^{2+} concentrations (pH 7.5, 25 °C) for the monophasic cleavage reaction (k) or the faster first phase of the biphasic reaction (k_1) are shown for the derivatives of clones 25, 28 and 34 (Rz25a, Rz28a and Rz34a) in the context of Figure 3. Clone 34

(derivative Rz34a) with the UUUU loop turned out to be the most active variant; it cleaved substrate II with very high k_1 values of 12.08 min^{-1} (pH 8.0, 37 °C) and 4.53 min^{-1} (pH 7.5, 25 °C) at 10 mM Mg^{2+} concentration, and $k_1 = 1.83\,min^{-1}$ (pH 8.0, 37 °C) and $k = 0.57\,min^{-1}$ (pH 7.5, 25 °C) at 1 mM Mg^{2+} concentration (Table 1, Rz34a).

Ribozyme activity with different substrates

In order to analyse clone 34 (Rz34) in a different sequence context we chose the well-characterised HH16 substrate which has become a standard in hammerhead ribozyme kinetics,[14, 15] and which has an extended eight-bp stem I (Figure 4). The appropriate derivative of Rz34 (Rz34b in Figure 4, Table 1) cleaved this substrate with $k = 0.34\,min^{-1}$ at 1 mM Mg^{2+} concentration and with $k_1 = 2.99\,min^{-1}$ at 10 mM Mg^{2+} concentration (both pH 7.5, 25 °C).

We further tested the activity of Rz34 in the context of substrate KrS17 (Table 1, derivative Rz34c) used in the selection study of Conaty et al.[13] The combination of Rz34 and KrS17 showed the fastest cleavage rate in this study ($k = 0.88\,min^{-1}$ at 1 mM Mg^{2+} concentration, pH 7.5, 25 °C) with an endpoint of about 90%. Based on the finding that a change of the reaction conditions from pH 7.5/25 °C to pH 8.0/37 °C increased the cleavage rate at 1 mM Mg^{2+} concentration about threefold (Table 1, see Rz25a, 28a and 34a), this indicates that Rz34 acts on substrate KrS17 at least twofold faster than any ribozyme variant selected by Conaty et al.[13] In conclusion, our results demon-

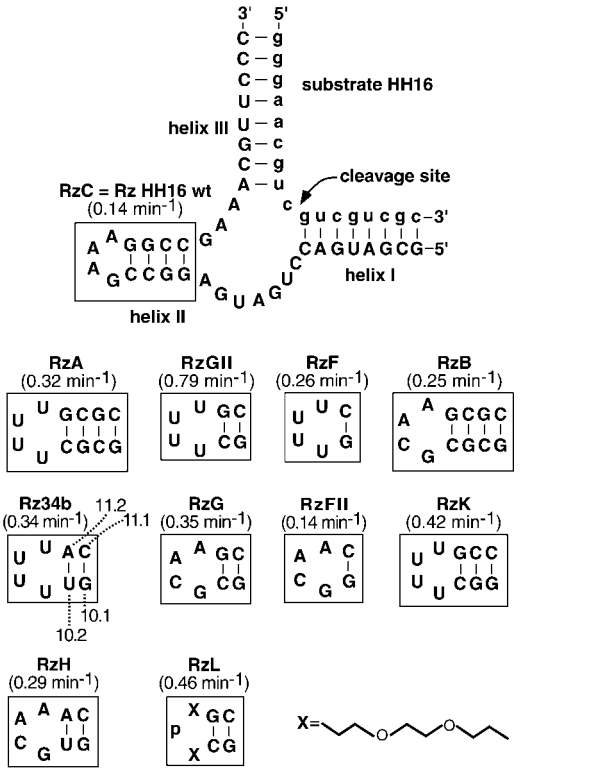


Figure 4. Tested variants of HH16-type ribozyme – substrate complexes that only differ in their stem-loop II structure. RzC is identical to the canonical HH16 ribozyme and is defined here as the "wild-type" (wt). The HH16 substrate is shown in lower case letters. Single-turnover rates obtained at 1 mM Mg^{2+} ions, pH 7.5 and 25° C are given in parentheses for each ribozyme variant.

Table 1. *Kinetic parameters for selected ribozymes at 1 mM and 10 mM Mg^{2+} concentrations.*

Stem-loop II[a]	Rz[b]	Substrate[b]	pH	T [°C]	k [min^{-1}], (F_∞ [%]) for s-exp[c]		k_1 [min^{-1}], [F_1 [%]], (F_∞ [%]) for d-exp[d]	
					1 mM Mg^{2+}	10 mM Mg^{2+}	1 mM Mg^{2+}	10 mM Mg^{2+}
GCGCUUUUGCGC	A	HH16	7.5	25	0.32 (67.4)			1.79 [40.6] (73.0)
GCGCGCAAGCGC	B	HH16	7.5	25	0.25 (62.0)			1.22 [37.6] (54.7)
GGCCGAAAGGCC	C (= HH16 wt)	HH16	7.5	25	0.14 (79.7)	0.57 (60.5)		
GUUUUUAC	34b	HH16	7.5	25	0.34 (78.5)			2.99 [38.4] (82.3)
GUGCAAAC	H	HH16	7.5	25	0.29 (72.6)			1.90 [35.0] (79.5)
GCUUUUGC	GII	HH16	7.5	25	0.79 (78.8)			4.41 [48.4] (77.1)
GCGCAAGC	G	HH16	7.5	25	0.35 (73.6)	1.37 (59.3)		
GUUUUC	F	HH16	7.5	25	0.26 (77.7)			1.80 [40.7] (65.4)
GGCAAC	FII	HH16	7.5	25	0.14 (72.3)	0.21 (77.6)		
GGCUUUUGCC	K	HH16	7.5	25	0.42 (75.5)			3.66 [52.0] (68.8)
GCXpXGC	L	HH16	7.5	25	0.46 (78.7)			2.69 [41.0] (83.3)
GGCCGAAAGGCC	C (= HH16 wt)	HH16-GUA	7.5	25	0.10 (77.3)			0.72 [58.3] (83.3)
GUUUUUAC	34b	HH16-GUA	7.5	25	0.20 (76.0)	1.05 (72.9)		
GCUUUUGC	GII	HH16-GUA	7.5	25	0.48 (73.9)			3.05 [52.6] (70.3)
GUUUUUAC	34c	KrS17	7.5	25	0.88 (88.5)			13.10 [79.5] (87.9)
GUUUUUAC	34a	substrate 2	7.5	25	0.57 (74.6)			4.53 [64.6] (80.2)
GUUUUUAC	34a	substrate 2	8.0	37			1.83 [68.0] (91.0)	12.08 [69.8] (91.1)
GUCAUUAC	28a	substrate 2	7.5	25	0.10 (76.1)	0.58 (60.2)		
GUCAUUAC	28a	substrate 2	8.0	37	0.27 (80.1)			2.95 [34.6] (83.1)
GUUUACAC	25a	substrate 2	7.5	25	0.23 (75.1)			2.26 [71.4] (82.6)
GUUUACAC	25a	substrate 2	8.0	37	0.67 (80.7)			11.10 [65.6] (87.1)

[a] Nucleotides forming stem II are underlined. [b] RzC is defined as the "wild type" ribozyme (HH16 wt). Ribozymes 34a, 34b and 34c only differed in their stem I and III sequences specific for the respective substrate. HH16: 5′-GGG AAC GUC GUC GUC GC. HH16-GUA: 5′-GGG AAC GUA GUC GUC GC (cleavage occurs on the 3′ side of the triplet GUA). KrS17: 5′-UUG CGA GUC CAC ACU GG[15]. Substrate II: 5′-GGG AAC GUC AGC UC. Errors between individual experiments for the same ribozyme – substrate combination were typically 20%. [c] s-exp, single exponential: $F_t = F_\infty(1 - e^{k \cdot t})$; F_t = fraction of product at timepoint t, F_∞ = fraction of product at the endpoint of the reaction. [d] d-exp, double exponential: $F_t = F_1(1 - e^{k_1 t}) + F_2(1 - e^{k_2 t})$; F_1, F_2 = fraction of product at the endpoint of the first phase (F_1) and second phase (F_2) of the reaction, respectively.

strate that the most active selected ribozyme (clone 34) acts on different substrates with comparably high efficiency.

Importance of loop II

To probe the effect of loop II we changed the UUUU sequence in Rz34 to GCAA (RzH), one of the stable GNRA-type tetraloop sequences (where N = any nucleotide and A = purine; Figure 4). RzH cleaved the HH16 substrate at a rate of 0.29 min^{-1} (k) at 1 mM Mg^{2+} concentration and 1.90 min^{-1} (k_1) at 10 mM Mg^{2+}. Thus, replacing the UUUU with the GCAA tetraloop led to a reduction in activity (Table 1). To probe the importance of the U – A base pair 10.2 – 11.2 in stem II as obtained from the selection process, it was changed to C – G in combination with the UUUU loop (RzGII). This ribozyme, although previously identified to be very active at 10 mM Mg^{2+} concentration, had never been analysed at lower concentrations.[16] In the present study it turned out to be remarkably active at concentrations of 1 mM Mg^{2+} ions (k = 0.79 min^{-1}). Again activity was markedly reduced when the loop was changed to GCAA (RzG, Table 1). A ribozyme with a nonnucleotide linker[17] in place of loop II (RzL, Figure 4) showed activity intermediate between RzGII and RzG at 1 mM Mg^{2+} concentration. A variant with three base pairs in stem II (RzK) showed about half the activity of RzGII at a concentration of 1 mM Mg^{2+} ions with retention of high activity at 10 mM Mg^{2+}.

Conaty et al.[13] published a very similar selection study with the same aim in mind, also following the procedure of Ishizaka et al.[12] These authors also randomised 18 nucleotides in the stem-loop II region. In contrast to us, they obtained variants of the hammerhead ribozyme with stem II consisting of only a single G – C base pair 10.1 – 11.1. In order to have a direct comparison of the effect of one or two base pairs in stem II, we determined the activity of RzF (Figure 4, Table 1), which was identical to their ribozyme 6.21, against the HH16 substrate. At 1 mM Mg^{2+} concentration, RzF showed only one-third of the activity of RzGII with two base pairs. In further contrast to the previous study,[13] all hammerhead variants identified in our selection had the potential to form a second base pair in stem II in addition to the G – C base pair 10.1 – 11.1 (Figure 2). Thus, the suggestion by Conaty et al. that one base pair in stem II is optimal for activity at low Mg^{2+} concentrations is not tenable, at least for their KrS17 substrate (see above) and the HH16 substrate. We have clearly demonstrated for the HH16 substrate (Table 1) that two base pairs are more favourable than either one, three or four. This is further supported by a study of Tuschl and Eckstein.[16]

Exchanging UUUU in loop II of RzF for GCAA further reduced activity (RzFII). This agrees with data by Conaty et al. who had also reported that a pyrimidine-rich loop II is considerably more active than any other.[13] This is underlined by a comparison of the activity of RzB and RzC where the GAAA-containing structure is even less active than that with the GCAA motif, and both are inferior to RzA with the UUUU loop. This indicates that a tetra-U loop, but not a purine-rich loop, contributes to the formation of productive ribozyme – substrate complexes. In the same hairpin sequence context a GAAA tetraloop is thermodynamically more stable than a tetra-U loop.[18] Thus the tetra-U loop might represent a compromise between sufficient stability and a required conformational flexibiliy in the ribozyme structure.

Compared with the standard HH16 ribozyme, RzC, the increased activities of ribozyme variants RzA, RzB, Rz34b, RzH, RzGII, RzG, RzF, RzK and RzL at 1 mM Mg^{2+} concentration were accompanied by enhanced cleavage rates at 10 mM Mg^{2+} concentration. Also, high activities observed at 1 mM Mg^{2+} concentration are not restricted to cleavage at the 3′ side of GUC, but also apply to cleavage at the 3′ side of GUA, as documented by the activities of RzC, Rz34b and RzGII with substrate HH16 – GUA (Table 1).

Limitations of selection

Interestingly, the selection had not produced the particularly active ribozyme RzGII but rather Rz34 with a U – A base pair 10.2 – 11.2 instead of a C – G base pair. It is not clear at present why RzGII did not pass the selectivity screen. However, one possible explanation could be that reverse transcriptase deletes stem-loop II in the presence of a more stable stem II with two alternating G – C pairs, thus eliminating these variants in the course of the selection. The fact that a hammerhead structure was selected is not surprising as, within the sequence space chosen for selection, the hammerhead ribozyme is optimal for NUH cleavage (where N = any nucleotide and H = A, C or T).[19] The comparison of our results with those of Conaty et al., however, is interesting in that the two essentially identical selection protocols did not produce the same results.[13] All the clones they sequenced could only form a single stem II C – G base pair, whereas all clones we identified had the potential to form an additional U – A base pair. This is difficult to explain and may be due to the different helix I and III structures used for selection or subtle differences in the handling of the protocol, particularly in the sequence of Mg^{2+} concentrations applied to increase stringency. It is worthy of note that neither protocol found the most active ribozyme sequence.

Role of stem-loop II in the cleavage process

It is difficult to understand how a shorter stem II can exert an influence on the dependence of activity on Mg^{2+} concentration. A length dependence of stem II on activity has been reported previously.[20] Extension of stem II to more than four nucleotides had lowered activity. It was initially thought that an interaction with stem I might be responsible for this effect, but as the lower activity was found to be independent of stem I length, this was considered an unlikely interaction. An optimal combination of sufficient stability and a required conformational flexibility of the stem-loop II structure may provide a clue towards understanding the high cleavage rates of the "mini-ribozyme" variants. Several studies have pointed to a considerable structural rearrangement in the hammerhead ribozyme in order to achieve the transition state.[21] This may result in the spatial juxtaposition of the scissile phosphodiester and a metal ion bound to the phosphate of nucleotide 9 (P9) and base G10.1, although structure-based evidence for such a comprehensive conforma-

tional change is still missing.[21] Translocation of a P9/G10.1-bound Mg^{2+} ion to the scissile phosphodiester group may be one solution towards increasing cleavage efficiency, particularly at low Mg^{2+} concentrations. A stem II with two alternating G – C base pairs closed by a tetra-U loop may be optimal with respect to lowering the energy barrier for such a catalytically important structural rearrangement.

Kinetic behaviour

At an Mg^{2+} concentration of 1 mM (pH 7.5, 25 °C), ribozyme cleavage rate constants could be obtained by fitting the data to a single exponential with calculated endpoints between 75 and 90%. Under these conditions, ribozymes were kinetically "well-behaved".[22] The combination of Rz34 and substrate KrS17 was most efficient ($k = 0.88\ min^{-1}$, endpoint approximately 90%), thus representing a variant that shows the same cleavage efficiency as a "well-behaved" standard hammerhead ribozyme at a tenfold higher Mg^{2+} concentration of 10 mM. At 10 mM Mg^{2+} concentration, the majority of ribozymes analysed here showed biphasic kinetics, with a fast first and a slow second reaction phase (Table 1); this suggests that the higher Mg^{2+} concentration may stabilise slow-cleaving alternate conformations of the ribozyme – substrate complex. Apparently, ribozymes with fast and "well-behaved" kinetics at a concentration of 1 mM Mg^{2+} ions tend to deviate from simple kinetic behaviour at 10 mM Mg^{2+} concentration, conditions at which the rates of the first reaction phase become extremely fast and in several cases difficult to measure manually. Pronounced biphasic kinetics at 10 mM Mg^{2+} concentration may be related to a higher Mg^{2+} affinity of the ribozymes selected for efficient cleavage at low Mg^{2+} concentrations.

Conclusions

We have identified hammerhead ribozymes with two base pairs in stem II and preferably a tetra-U loop II that are considerably more active at lower Mg^{2+} concentrations than conventional ribozymes. They are also more active than the previously isolated "mini-ribozymes" with one base pair in stem II.[13] The ribozymes characterised should be advantageous for in vivo applications for the inhibition of gene expression, particularly under conditions where the hammerhead ribozyme cleavage reaction is not simply limited by the ribozyme – substrate association rate.

Experimental Section

Oligonucleotides: Oligonucleotides were synthesised on an Applied Biosystems 394A DNA synthesiser. DNA/RNA phosphoramidite monomers were supplied by Perseptive (Germany). Deprotection and purification were performed as described by Tuschl and Eckstein.[16] Gel-purified oligonucleotides were dissolved in double-destilled water and their concentrations were determined by UV spectroscopy. Samples were stored at −20 °C. For generation of pool 0 dsDNA primers A and B were synthesised: primer A: 5′-TGGTGCAAGCT***TAATACGACTCACTATA***GGGTTAAGCATCCTCGAGCT-3′; primer B: 3′-CCCAATTCGTAGGAGCTCGA (N)18 TGAGGCGACGTCT-TAAGCTCT-5′ (the T7 promotor site is shown in bold and the restriction sites (HindIII, PstI and EcoRI) are underlined). For the selection procedure a 26-mer RNA substrate was synthesised with a biotin linked at the 5′-end: 5′-Biotin-CAAGGAGUCAGCUCGAGGAUG-CUUAA-3′ (the cleaving-site triplet is underlined). For PCR amplification of the dsDNA primers C and D were used: primer C: 3′-TGAGGCGACGTCTTAAGCTCT-5′; primer D: 5′-TGGTGCAAGCTTAA-TACGACTCA-3′.

Preparation of Pool 0: Primer A and primer B (2.5 μM) were incubated in sequenase buffer (40 mM tris(hydroxymethyl)aminomethane (Tris)/HCl (pH 7.5), 20 mM $MgCl_2$, 50 mM NaCl) at 90 °C for 5 min, and then cooled to RT. 1,4-dithiothreitol (DTT; final concencentration 5 mM), deoxynucleoside triphosphates (dNTPs; final concentrations 375 μM each) and T7 sequenase v2.0 enzyme (0.08 U μL^{-1}; Amersham) were added and the mixture was incubated at 37 °C for about 1 h in a total volume of 500 μL. DNA was purified with Microcon spin columns (Millipore) and analysed on an agarose gel (Metafor, 2.5%).

T7 transcription: dsDNA (1 μM) was transcribed essentially as previously described.[9] After gel electrophoresis, full-length transcripts were excised from the gel and eluted by the crush-and-soak method in NaOAc (1 M; pH 4.6).

Selection procedure: The randomised pool RNA and the RNA – biotin substrate (1.5 equiv) were heated at 90 °C for 3 min, then incubated at 37 °C for 5 min and cooled to RT (about 10 min).

Binding of biotinylated RNA to Dynabeads M280 Streptavidin (DYNAL): a) Dynabeads were washed with washing buffer (10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES, pH 6.8), 5 mM ethylenediaminetetraacetate (EDTA), 50 mM NaCl); b) annealed pool RNA/RNA – biotin complexes were mixed with washed Dynabeads in binding buffer (10 mM HEPES (pH 6.8), 5 mM EDTA, 100 mM NaCl) and kept at RT under gentle mixing for about 20 min. The supernatant was removed and the beads were washed with washing buffer.

Cleavage reaction: The beads were suspended in cleavage buffer (50 mM Tris/HCl (pH 7.5), 50 mM NaCl) containing either 20 or 1 mM Mg^{2+} ions (see below) and the suspension was incubated at 37 °C. After gentle mixing the supernatant was decanted and the RNA was concentrated by ethanol precipitation. The amount of RNA, incubation time and concentration of Mg^{2+} ions in the various selection rounds: 0 →1: 1.5 nmol (33 μM) RNA, 8 min, 20 mM Mg^{2+}; 1 →2: 440 pmol (8.8 μM) RNA, 8 min, 20 mM Mg^{2+}; 2 →3: 440 pmol (2.5 μM) RNA, 8 min, 20 mM Mg^{2+}; 3 →4: 440 pmol (2.2 μM) RNA, 8 min, 20 mM Mg^{2+}; 4 →5: 440 pmol (3.9 μM) RNA, 2 min, 1 mM Mg^{2+}; 5 →6: 440 pmol (4.7 μM) RNA, 1 min, 1 mM Mg^{2+}; 6 →7: 440 pmol (4.9 μM) RNA, 30 sec, 1 mM Mg^{2+}; 7 →8: 440 pmol (5.4 μM) RNA, 30 sec; 1 mM Mg^{2+}.

Reverse transcription: Selected RNAs and primer C (1.1 equiv) were heated in H_2O to 90 °C for 3 min and cooled to RT. This was followed by reverse transcription for 1 h at 37 °C in reaction buffer (50 μL; 50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT) containing each dNTP (0.5 mM) and Superscript RNase H^- RT (10 U μL^{-1}; GIBCO BRL). The mixture was then heated to 75 °C and cooled to 4 °C. NaOH was added (final concentration 200 mM) and the RNA was hydrolysed by incubation for 1 h at 37 °C. The cDNA was concentrated by ethanol precipitation in the presence of NH_4OAc.

Restoration of the T7 promotor: dsDNA was produced from the single-stranded cDNA in a separate reaction. For this purpose, cDNA was annealed to primer A and reacted with sequenase as described above but in a total volume of 100 μL. The dsDNA from the sequenase reaction was amplified by PCR as follows: primer C and D

(0.5 μM), each dNTP (0.2 mM), $MgCl_2$ (1.5 mM) and Taq DNA Polymerase (0.025 U μL^{-1}; Amersham Pharmacia) were incubated in Taq reaction buffer (not specified by Amersham) for 10 – 25 cycles in a total volume of 100 μL. Cycling parameters were as follows: step 1, 95 °C for 1 min; step 2, 65 °C for 2 min; step 3, 72 °C for 1 min. Before starting the first PCR cycle the reaction was preheated at 95 °C for 30 s. PCR products were concentrated by ethanol precipitation.

Cloning and sequencing: Pool 8 DNA was loaded on a 3% agarose gel, and the main band was excised and eluted by using the JetSorb Gel Extraction Kit/150 (Genomed). This solution (1 μL) was amplified by PCR as described above and directly used for cloning with the TOPO TA Cloning Kit (Invitrogen). 25 white and 25 blue colonies were picked and purified by Qiagen-20 miniprep columns.

Cleavage kinetics: Single-turnover kinetics were performed with chemically synthesised ribozymes (500 nM) and substrates (25 nM) in 50 mM Tris/HCl at pH 7.5 and 25 °C (or pH 8.0 and 37 °C), in the presence of 1 or 10 mM $MgCl_2$ as described previously.[22, 23] The nonnucleotide linker of RzL was synthesised by using the spacer phosphoramidite 9 obtained from Glen Research. ^{32}P-labelled substrate and product bands were analysed on 20% polyacrylamide gels containing 8 M urea, and visualised and quantified with a Bio-Imaging Analyzer BAS-1000 or -2000 (Fujifilm) and the analysis software PCBAS/AIDA (Raytest). Pseudo-first-order rate constants of cleavage (k or k_1, k_2) were calculated by nonlinear regression analysis (with the programs Grafit 3.0, Erithacus Software, and KaleidaGraph 3.0); the data were fitted to the equation for a single exponential [Equation (1)] or a double exponential [Equation (2)], where F_t = fraction of substrate cleaved, t = time, F_∞ = fraction of substrate cleaved at the endpoint and F_1, F_2 = fraction of substrate cleaved at the endpoint of the first and second phase of the reaction, respectively.

$$F_t = F_\infty \times (1 - e^{-kt}) \quad (1)$$

$$F_t = F_1 \times (1 - e^{-k_1 t}) + F_2 \times (1 - e^{-k_2 t}) \quad (2)$$

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