Volume 17 Number 4 1989 Nucleic Acids Research

A catalytic 13-mer ribozyme

Alex C.Jeffries and Robert H.Symons*

Department of Biochemistry, University of Adelaide, Adelaide, SA 5000, Australia

Received January 3, 1989; Accepted January 26, 1989

ABSTRACT

A 13-mer oligoribonucleotide can act as a ribozyme for the specific self-cleavage of a 41-mer oligoribonucleotide substrate in the presence of Mg^{2+}. The two sequences involved correspond to the self-cleavage hammerhead structure of the virusoid of lucerne transient streak virus. The Michaelis-Menten kinetic parameters for the reaction were; Km 1.3 μM, Vmax 0.012 μM min^{-1}, kcat 0.5 min^{-1}. The 13-mer RNA is the smallest ribozyme so far reported. A DNA analogue of the 13-mer can not substitute for the RNA in the reaction.

INTRODUCTION

Certain small RNA molecules pathogenic to flowering plants are able to undergo site specific cleavage of their phosphodiester backbones in the presence of a divalent metal ion but in the absence of proteins. This self-cleavage reaction generates 5'-OH and 2',3'-cyclic phosphodiester termini and is believed to be an integral step in the proposed rolling-circle replication mechanism of these RNAs, whereby longer-than-unit-length RNAs are self-cleaved to give monomeric units (1, 2).

Self-cleavage of the plus and minus RNA transcripts from cDNA clones of the satellite RNA of tobacco ringspot virus (sTRSV), avocado sunblotch viroid (ASBV), and the virusoid of lucerne transient streak virus (vLTSV) has been demonstrated previously (3-6). A high degree of primary (13 nucleotides) and secondary structure homology about the self-cleavage sites of these RNAs has been observed, with the exception of minus sTRSV (4,6), and has led to the proposal of a hammerhead structure to help explain the structural requirements for these reactions. This model has been confirmed by deletion analyses whereby a 52 nucleotide RNA molecule corresponding to a single plus vLTSV hammerhead was shown to undergo rapid and specific self-cleavage (7). Recently, an RNA transcript from a 330 base-pair tandemly repeated satellite DNA sequence in the genome of the newt (*Notophthalmus viridescens*) has been shown to self-cleave *in vitro* (8). A hammerhead structure containing the 13 conserved nucleotides can also be drawn around this cleavage site. However, in the case of ASBV and the newt sequences, stable self-cleavage structures are formed by the association of two self-cleavage sites to form double-hammerheads (9).

The hammerhead structures for plus and minus ASBV differ from other hammerheads in

that their constituent nucleotides are drawn from two regions which are separated by at least 88 nucleotides (plus) or 77 nucleotides (minus) in the primary structure of the 247 nucleotide viroid (5). With all other hammerheads, including those proposed for the virusoids of velvet tobacco mottle virus, solanum nodiflorum mottle virus and subterranean clover mottle virus (6,10), the nucleotides constituting the hammerhead come from a relatively small region of contiguous nucleotides in the primary structure.

The essentially binary nature of the ASBV hammerheads indicated that they could be active in the form of two separate molecules and this was shown by specific self-cleavage when two large fragments of minus ASBV were hybridized together (11). That one of these molecules could act as a catalyst if mixed with substrate molecules under conditions which favour cycles of melting and annealing was demonstrated in a simpler system developed by Uhlenbeck (12, Fig. 1b). In a similar way, the hammerhead of plus sTRSV has been separated into two components and shown to cleave in a catalytic manner (13, Fig. 1c). The hammerhead from the newt satellite DNA transcript and a hammerhead which shows no sequence homology with known hammerheads, except in that it contains the 13 conserved nucleotides, have also been split into two and three separate molecules (Fig. 1d), respectively, and shown to self-cleave (14, 15); however, since in both of these cases the putative enzymes were in a 1.5-fold excess over substrate, it was not demonstrated that they worked in a catalytic manner.

Here we describe an alternative catalytic system using plus vLTSV sequences in which a 13-mer Ribozyme (R) acts as a catalyst on a 41-mer Substrate RNA (S) (Figs. 1a and 2a). This is the smallest ribozyme yet reported.

MATERIALS AND METHODS

Preparation of RNAs

Synthetic oligodeoxyribonucleotides were prepared on an Applied Biosystems Model 380B DNA synthesizer (Bresatec, Adelaide) and full-length products purified by 90 mM Tris-borate, pH 8.3, 2 mM EDTA (TBE)/7M urea, 10% polyacrylamide gel electrophoresis, elution and ethanol precipitation. RNAs were produced by transcription of a single-stranded DNA template using T7 RNA polymerase as first described by Milligan et al. (16). Individual templates and transcription primer were annealed together in a one-to-one molar ratio in 10 mM Tris-HCl, pH 7.0, by heating at 65°C for 3 minutes followed by snap-cooling on ice. Non-radioactive transcription reactions contained 0.1 pmole/µl DNA template, 40 mM Tris-HCl, pH 8.1, 12 mM $MgCl_2$, 10 mM DTT, 2 mM of each NTP, 1 mM spermidine, 0.01% triton X-100, 0.05 µg/µl BSA, 1 U/µl RNasin (Promega) and 10 U/µl T7 RNA polymerase (Bresatec, Adelaide). Radioactive transcription reactions differed only in having 1 mM ATP, CTP and GTP, 0.025 mM UTP, 1 U/µl T7 RNA polymerase and up to 2 µCi/µl α-^{32}P-UTP (Bresatec, Adelaide). Full-length transcripts were purified by TBE/7M urea, 15% polyacrylamide gel electrophoresis, elution and either ethanol or ethanol:acetone (2:1, v/v) precipitation in the

presence of 0.3 M sodium acetate. Sequences were checked by enzymic methods essentially as described in Haseloff and Symons (17). RNA concentrations were estimated by UV spectroscopy or liquid scintillation counting.

Self-cleavage reactions

In a standard self-cleavage reaction, non-radioactive 13-mer (Ribozyme, R) and 41-mer (Substrate, S) RNAs (Figs. 1a and 2a) were mixed together at room temperature in 1 mM sodium EDTA at molar ratios ranging from 1:1 to 1:217. Included in all reactions was 1 ng of ^{32}P-S which allowed the reactions to be followed by autoradiography and to be quantitated by liquid scintillation counting. Self-cleavage buffer was then added to give final conditions in 10 µl of 0.5 mM sodium EDTA, 50 mM Tris-HCl, pH 8.5, 20 mM $MgCl_2$. Control reactions did not contain Mg^{2+}. Solutions were incubated at 50°C under liquid paraffin for the appropriate times. All reactions were terminated by the addition of an equal volume of 95% formamide, 10 mM sodium EDTA, 0.02% xylene cyanol FF, 0.02% bromophenol blue. Cleavage fragments and residual full-length RNAs were resolved by TBE/7M urea, 20% polyacrylamide gel electrophoresis and the extent of cleavage estimated by excision of gel slices and scintillation counting in triton-toluene scintillation fluid.

RESULTS

To test the possibility of catalytic self-cleavage using the plus vLTSV hammerhead sequence, two small RNAs (S and R) were produced with sequences permitting base-pairing and formation of the secondary structure shown in Figs. 1a and 2a. The sequences used were the same as wild-type vLTSV-A (18) except for the GGG sequence at the 5'-end of S, which was dictated by the promoter requirement, on the DNA template, of T7 RNA polymerase (16). With this system, cleavage at the site indicated by the arrow (Figs. 1a and 2a) would produce a 9 nucleotide 5'-cleavage fragment (5'F) and a 32 nucleotide 3'-cleavage fragment (3'F).

When S and R were mixed together in a 2:1 molar ratio at room temperature and

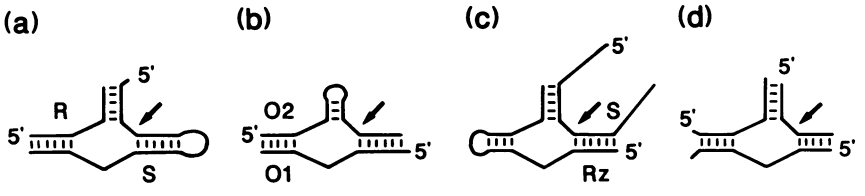

Fig. 1. Schematic diagram of the putative hammerhead secondary structures of four ribozyme systems. (a) Putative hammerhead secondary structure of ribozyme (R) and substrate (S) RNAs based on the sequence of plus vLTSV-A (18). (b) Putative hammerhead secondary structure of ribozyme (O1) and substrate (O2) RNAs based on the sequence of minus ASBV, as described by Uhlenbeck (12). (c) Putative hammerhead secondary structure of ribozyme (Rz) and substrate (S) RNAs based on the sequence of plus sTRSV, as described by Haseloff and Gerlach (13). (d) Putative secondary structure of three-way hammerhead as described by Koizumi et al. (15). In all figures arrows and dashes indicate the site of self-cleavage and base-pairs, respectively.

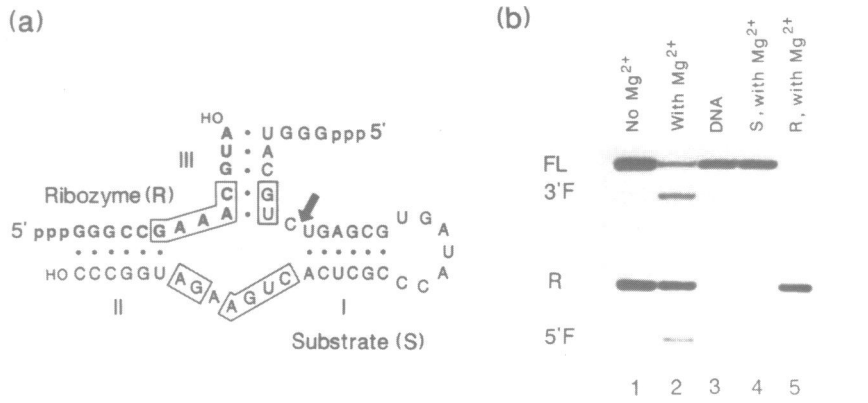

Fig. 2. Self-cleavage by plus vLTSV ribozyme system. (a) Primary and putative hammerhead secondary structure of Ribozyme (R) and Substrate (S) RNAs. All sequence except GGG at 5'-end of S are from plus vLTSV-A (18). Boxed nucleotides, roman numerals (I, II and III) and arrow indicate the 13 nucleotides, stems and cleavage site, respectively, that are conserved among hammerhead structures (6,10). (b) Denaturing polyacrylamide gel electrophoresis of catalytic self-cleavage of S by R. Lane 1; R and S RNA incubated with no Mg^{2+}. Lane 2; R and S RNA incubated with Mg^{2+}. For lanes 1 and 2 concentrations of S and R where 0.046 μM and 0.023 μM, respectively. That is, the S:R molar ratio was 2:1. Lane 3; DNA 13-mer and S RNA incubated as in lane 2 except DNA:S molar ratio was 10:1. Lane 4; S RNA incubated as in lane 2. Lane 5; R RNA incubated as in lane 2. All reactions incubated at 50°C for 6 hours. FL; full-length S. 5'F; 5' cleavage fragment (9 nucleotides). 3'F; 3' cleavage fragment (31 nucleotides). R; as in (a).

subjected to self-cleavage conditions (see above) for 6 hours, self-cleavage occurred (Fig. 2b, lane 2). Furthermore, R was able to direct the cleavage of more than one S molecule as virtually total cleavage of S was obtained with longer incubation times (from 10 to 24 hours, data not shown). Sizing, enzymatic sequencing (17) and terminal nucleotide analysis of cleavage fragments (5, 6) showed that cleavage occurred at the expected site (Figs. 1a and 2a) and that 5'-OH and 2',3'-cyclic phosphodiester termini were generated during the self-cleavage reactions (data not shown). As with other hammerhead self-cleavage reactions, Mg^{2+} was an essential requirement for the reaction (Fig. 2b, lanes 1, 2, 4 and 5). Heating and either snap-cooling on ice or slow-cooling of R and S RNAs before addition of self-cleavage buffer did not change the extent or initial velocity of the subsequent self-cleavage reaction as compared to when the two

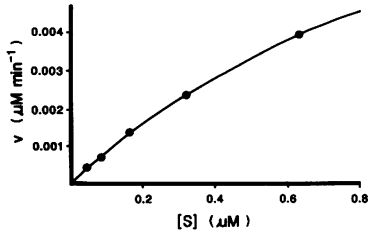

Fig. 3. Non-linear regression plot of initial velocity (v) for cleavage of S by R versus S concentration ([S]). Concentrations of S ranged from 0.05 to 0.6 μM while the concentration of R was constant at 0.023 μM. Data was fitted directly to the Michaelis-Menten equation (20) using an algorithm adapted from that of Duggleby (21). Initial values for Km and Vmax were obtained from Eadie-Hofstee plots (20) of the same data using a linear regression algorithm developed by Filip Lim (pers. comm.). This plot fits Km and Vmax values of 1.3 μM and 0.012 μM min^{-1}, respectively.

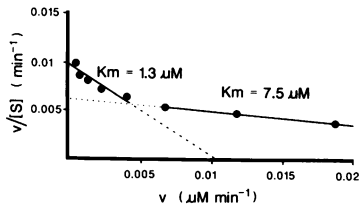

Fig. 4. Eadie-Hofstee plots of kinetic data. Two Eadie-Hofstee plots (20) were superimposed to illustrate the deviation from linearity of the data for S concentrations ranging from 0.05 to 5 μM while R concentration remained constant at 0.023 μM. Km values of 1.3 and 7.5 μM fit S ranges of 0.05 to 0.6 μM and 0.6 to 5 μM, respectively.

RNAs were mixed at room temperature (data not shown). Since R was not changed during these self-cleavage reactions (Fig. 2b, lanes 1, 2 and 5) it was concluded that, under the conditions used, R behaved as a catalyst.

A DNA analogue of R could not direct self-cleavage of S even when in a 10-fold molar excess over S (Fig. 2b, lane 3). A wide variety of pHs, temperatures and ionic concentrations known to cause self-cleavage of S by R were also unable to cause self-cleavage of S by DNA (data not shown).

Time course analyses of self-cleavage using 1:1 molar ratios of R:S at temperatures ranging from 20°C to 60°C were conducted and an Arrhenius plot (19) drawn which indicated that the optimal reaction temperature was approximately 50°C. When assuming a second-order reaction mechanism this plot gives an activation energy (Eact) for the reaction of 10.7 Kcal mol^{-1} (data not shown).

The Michaelis-Menten kinetic parameters (20) for this reaction were determined by conducting multiple self-cleavage reactions with concentrations of S varying from 0.05 μM to 5 μM while the concentration of R remained constant at 0.023 μM. Initial velocities for these reactions were plotted against the appropriate concentration of S and fitted directly to the Michaelis-Menten equation (20) using a non-linear regression program based on that of Duggleby (21, Fig. 3) and using estimates for Km and Vmax obtained from Eadie-Hofstee plots (20). The Km and Vmax were found to be 1.3 μM and 0.012 μM min^{-1}, respectively, for S concentrations ranging from 0.05 μM to 0.6 μM. These data correspond to a kcat of 0.5 min^{-1}. With S concentrations greater than 0.6 μM the Km and Vmax values changed to 7.5 μM and 0.05 μM min^{-1}, respectively. This is best illustrated by the Eadie-Hofstee plots in Fig. 4. These results were reproducible in repeated experiments using different preparations of RNA.

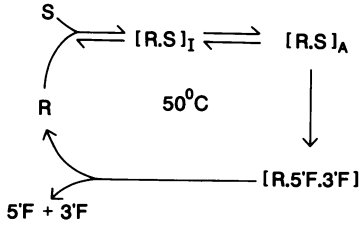

Fig. 5. Schematic diagram of the self-cleavage reaction of Substrate (S) catalyzed by Ribozyme (R). The diagram shows the putative equilibriums involved in the reaction and the recycling nature of R. R, S, 5'F and 3'F as described in Fig. 2. Square boxes represent various complexes between R, S and self-cleavage fragments. A and I indicate active and one or more inactive conformations, respectively.

Table 1.

Kinetic data for two hammerhead structure ribozymes

	vLTSV system	Uhlenbeck's system[1]
Km	1.3 μM	0.63 μM
Vmax	0.012 μM min^{-1}	0.0475 μM min^{-1}
kcat	0.5 min^{-1}	0.5 min^{-1}
Eact	10.7 Kcal mol^{-1}	13.1 Kcal mol^{-1}

[1] Based on minus ASBV sequence (12).

DISCUSSION

The relatively high incubation temperature of 50°C required for optimal self-cleavage in *trans* is most likely required to allow dissociation of R molecules after a round of cleavage has occurred, followed by reannealing to other S molecules (Fig. 5). Such a high temperature optimum was also observed for two similar catalytic RNA systems (12,13). In Fig. 5 we have proposed that the active conformation of R and S ($[R.S]_A$) is in equilibrium with one or more inactive conformations ($[R.S]_I$); this concept is similar to the active-inactive structural switch demonstrated around the self-cleavage site of vLTSV (6,11). The high temperature requirement for efficient cleavage could play a part in moving this equilibrium towards the active conformation as well as in causing the dissociation and reannealing of R to S molecules.

The Km, Vmax, kcat and Eact values reported here are similar to those reported for Uhlenbeck's system (12, Table 1). No kinetic data has been published for the three other *trans* self-cleavage systems (13-15, Fig. 1c and 1d).

However, what had not been reported before was the change from simple Michaelis-Menten kinetics as substrate concentration increased (Fig. 4). This may be partially explained by postulating that at high substrate concentrations, interactions between S molecules become significant and so increase the apparent dissociation constant of [R.S] complexes (Fig. 5), hence the higher Km of 7.5 μM. Therefore, in stating the kinetic parameters for our reaction as Km 1.3 μM and Vmax 0.012 μM min^{-1}, we have assumed that at concentrations of S below 0.6 μM, self-association between S molecules was negligible. The effect of higher S concentrations on the Michaelis-Menten kinetic parameters does not fit any of the classical types of inhibition (e.g., competitive, uncompetitive or non-competitive, 20).

The base-pairing requirement to form the active self-cleavage structure could be a reason why these reactions are slower than protein catalysed reactions which have kcat values ranging from the order of 10 to 10^8 min^{-1} (20) as compared to 0.5 min^{-1} as found here.

ACKNOWLEDGEMENTS

This work was supported by the Australian Research Grants Scheme and the Commonwealth Special Research Centre For Gene Technology. A. C. J. is supported by a

Commonwealth Postgraduate Research Award. We thank Dr. John Coates for helpful discussion on kinetic data, Dr. Filip Lim for computing advice, Candice Sheldon and Dr. Chris Davies for comments on the manuscript and Tammy Greatrex for technical assistance.

*To whom correspondence should be addressed

REFERENCES

1. Branch, A. D. and Robertson, H. D. (1984) Science **223**, 450-455.
2. Hutchins, C. J., Keese, P., Visvader, J. E., Rathjen, P. D., McInnes, J. L. and Symons, R. H. (1985) Plant Mol. Biol. **4**, 293-304.
3. Prody G.A., Bakos, J.T., Buzayan, J.M., Schneider, I.R. and Bruening, G. (1986) Science **231**, 1577-1580.
4. Buzayan, J. M., Gerlach, W. L. and Bruening, G. (1986) Nature **323**, 349-353.
5. Hutchins, C. J., Rathjen, P. D., Forster, A. C. and Symons, R. H. (1986) Nucl. Acids Res. **14**, 3627-3640.
6. Forster, A. C. and Symons, R. H. (1987) Cell **49**, 211-220.
7. Forster, A. C. and Symons, R. H. (1987) Cell **50**, 9-16
8. Epstein, L. M. and Gall, J. G. (1987) Cell **48**, 535-543.
9. Forster, A.C., Davies, C., Sheldon, C.C., Jeffries, A.C. and Symons, R.H. (1988) Nature **334**, 265-267.
10. Keese, P. and Symons, R. H. (1987) In Semancik, J. S. (ed), Viroids and Viroid-like Pathogens, CRC Press, Florida, pp. 1-47.
11. Forster, A. C., Jeffries, A. C., Sheldon, C. C. and Symons, R. H. (1987) Cold Spring Harb. Symp. Quant. Biol. **52**, 249-259.
12. Uhlenbeck, O. C. (1987) Nature **328**, 596-600.
13. Haseloff, J. and Gerlach, W. L. (1988) Nature **334**, 585-591.
14. Koizumi, M., Iwai, S. and Ohtsuka, E. (1988) FEBS Lett. **228**, 228-230.
15. Koizumi, M., Iwai, S. and Ohtsuka, E. (1988) FEBS Lett. **239**, 285-288.
16. Milligan, J. F., Groebe, D. R., Witherell, G. W. and Uhlenbeck, O. C. (1987) Nucl. Acids Res. **15**, 8783-8798.
17. Haseloff, J. and Symons, R. H. (1981) Nucl. Acids Res. **9**, 2741-2752.
18. Keese, P., Bruening, G. and Symons, R. H. (1983) FEBS Lett. **159**, 185-190.
19. Atkins, P. W. (1986) Physical Chemistry, 3rd edn, pp. 697-701. Oxford University Press, Oxford.
20. Fersht, A. (1977) Enzyme Structure and Mechanism, pp. 84-133.W. H. Freeman, San Francisco.
21. Duggleby, R. G. (1981) Anal. Biochem. **110**, 9-18.