Hammerhead-like Molecules Containing Non-Nucleoside Linkers Are Active RNA Catalysts

Fritz Benseler,‡ Dong-jing Fu,† Janos Ludwig,‡ and Larry W. McLaughlin*,†

Ribonetics, Goettingen, Germany
Department of Chemistry, Merkert Chemistry Center
Boston College, Chestnut Hill, Massachusetts 02167

Received June 15, 1993

The preparation of synthetic RNA-like molecules with catalytic activity similar to that of the hammerhead ribozyme,[1] but simpler to prepare than a complete RNA sequence, could be an important route to the generation of truncated catalysts amenable to detailed structural analyses by NMR or related methods. These simplified materials may also offer a novel and potentially valuable class of antiviral agents that will rely upon well-known base–base hydrogen-bonding interactions for sequence location and will then cleave the target RNA by transesterification of the scissle phosphodiester.[2] A single selective cleavage event in a target sequence, such as viral mRNA, will inactivate further biological processing of the transcript and should ultimately inhibit viral growth.

The consensus hammerhead RNA/substrate complex derived from plant viroid RNAs and proposed by Uhlenbeck[2] is composed of three helical stems, one of which terminates in a hairpin loop (see native structure, Figure 1). The nominally single-stranded core sequence, in conjunction with (a) metal (Mg^{2+} or Mn^{2+}) cofactor(s), appears to be responsible for the observed catalytic activity.[3] Nucleoside substitution studies[4] have indicated that the core sequence must be largely conserved in order to maintain efficient cleavage activity, while the sequences of stems I and III can be varied to permit effective sequence targeting of the substrate RNA. The use of nucleoside analogues at various sites within the complex has permitted the identification of specific functional groups within the core sequence that must be maintained for efficient catalysis.[5] While little structural information is available,[6] the core sequence is likely important in the formation of a specific secondary/tertiary structure necessary to position the requisite metal cofactor(s) for efficient transesterification of the phosphodiester in the target RNA. Other organic components could, in principle, be employed in place of the natural nucleoside building blocks and still permit the formation of an active catalyst.

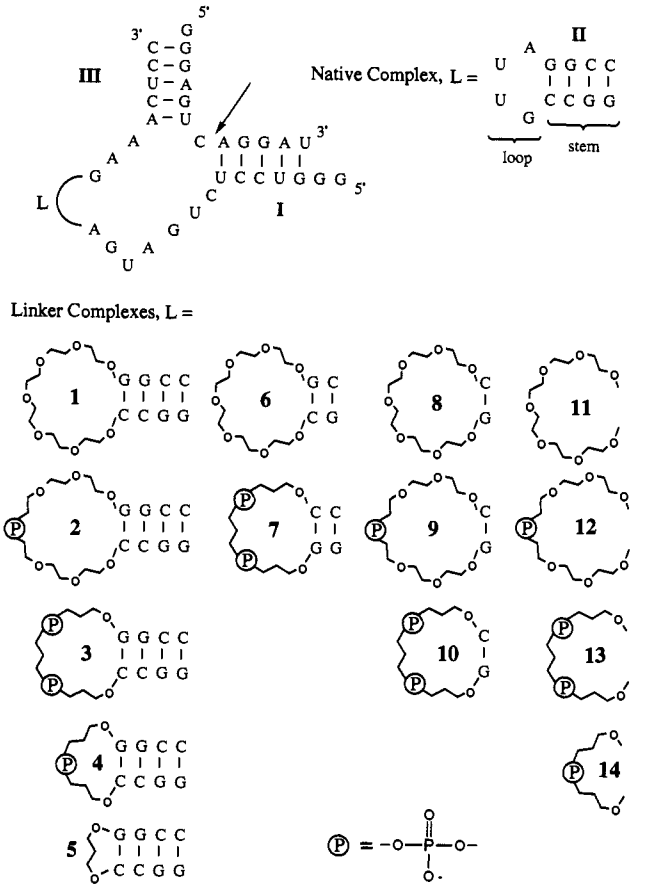

Figure 1. Structures of the of the native hammerhead RNA and hammerhead-like RNA-linker complexes. In the linker complexes, loop II and stem II have been replaced by the linker structures L, consisting of hexaethylene glycol (**1**, **6**, **8**, **11**), two molecules of triethylene glycol joined with a phosphodiester linkage (**2**, **9**, **12**), or one (**5**) or more (**3**, **4**, **7**, **10**, **13**, **14**) molecules of 1,3-propanediol joined with phosphodiester linkages, and four, two, one, or zero base pairs of the native stem. Both ribozyme and substrate sequences were prepared using supports containing 2′-deoxynucleosides. The arrow marks the substrate cleavage site.

RNA-based therapeutics suffer from at least two disadvantages: (i) they are labile to intracellular nucleases and thus unlikely to exert the desired targeting and cleavage activity during their short lifetimes within cells and (ii) therapeutics consisting of some 30 or more nucleoside residues will be difficult, if not expensive, to produce in quantities sufficient for use as pharmaceuticals. The synthesis of nuclease-resistant sequences by the incorporation of nucleoside analogues has been reported.[5e,7] But in order to maintain significant cleavage activity, some ribonucleosides must remain in the core sequence. Attempts to shorten the ribozyme sequence have had mixed results.[8]

Stem II of the ribozyme/substrate complex (see Figure 1, native sequence) does not appear to contain any critical nucleoside residues,[4e] but it is likely to play a role in defining the overall conformation of the active complex. Stem II and loop II cannot be eliminated from the ribozyme but in some cases can be replaced with shorter nucleotide sequences.[8b] In the present work, we have prepared truncated versions of the hammerhead complex in which the noncritical nucleoside residues of stem and loop II have been replaced in part or in full by simple synthetic organic

† Boston College.
‡ Ribonetics.

(1) For a recent review, see: Symons, R. H. *Annu. Rev. Biochem.* **1992**, *61*, 641–671, and references therein.

(2) (a) Uhlenbeck, O. C. *Nature* **1987**, *321*, 596–600. See also: (b) Sampson, J. R.; Sullivan, F. X.; Behlen, L. W.; DiRenzo, A. B.; Uhlenbeck, O. C. *Cold Spring Harbor Symp. Quant. Biol.* **1987**, *52*, 267–275.

(3) (a) Dahm, S. C.; Uhlenbeck, O. C. *Biochemistry* **1991**, *30*, 9464–9469. (b) Koizumi, M.; Otsuka, E. *Biochemistry* **1991**, *30*, 5145–5150.

(4) (a) Koizumi, M.; Ewai, S.; Otsuka, E. *FEBS Lett.* **1988**, *239*, 285–288. (b) Jefferies, A. C.; Symons, R. H. *Nucleic Acids Res.* **1989**, *17*, 1371–1377. (c) Ruffner, D. L.; Dahm, S. C.; Uhlenbeck, O. C. *Gene* **1989**, *82*, 31–41. (d) Fedor, M. J.; Uhlenbeck, O. C. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 1668–1672. (e) Ruffner, D. E.; Stormo, G. D.; Uhlebeck, O. C. *Biochemistry* **1990**, *29*, 10695–10702.

(5) (a) Koizumi, M.; Hayase, Y.; Iwai, S.; Kamiya, H.; Inoue, H.; Otsuka, E. *Nucleic Acids Res.* **1989**, *17*, 7059–7070. (b) Yang, J.-H.; Perreault, J.-P.; Labuda, D.; Usman, N.; Cedergren, R. *Biochemistry* **1990**, *29*, 11156–11160. (c) Perreault, J.-P.; Wu, T.; Cousineau, B.; Ogilvie, K. K.; Cedegren, R. *Nature* **1990**, *344*, 565–567. (d) Odai, O.; Hiroaki, H.; Sakata, T.; Tanaka, T.; Uesugi, S. *FEBS Lett.* **1990**, *267*, 150–152. (e) Pieken, W. A.; Olsen, D. B.; Benseler, F.; Aurup, H.; Eckstein, F. *Science* **1991**, *253*, 314–316. (f) Olsen, D. B.; Benseler, F.; Aurup, H.; Picken, W. A.; Eckstein, F. *Biochemistry* **1991**, *30*, 9735–9741. (g) Williams, D. M.; Pieken, W. A.; Eckstein, F. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 918–921. (h) Fu, D.-J.; McLaughlin, L. W. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 3985–3989. (i) Slim, G.; Gait, M. J. *Biochem. Biophys. Res. Commun.* **1992**, *183*, 605–609. (j) Yang, J.-H.; Usman, N.; Chartrand, P.; Cedegren, R. *Biochemistry* **1992**, *31*, 5005–5009. (k) Fu, D.-J.; McLaughlin, L. W. *Biochemistry* **1992**, *31*, 10941–10949.

(6) For preliminary NMR studies, see: (a) Heus, H. A.; Uhlenbeck, O. C.; Pardi, A. (1990) *Nucleic Acids Res.* **1990**, *18*, 1103–1108. (b) Heus, H. A.; Pardi, A. (1991) *J. Mol. Biol.* **1991**, *217*, 113–124.

(7) Paolella, G.; Sproat, B. S.; Lamond, A. I. *EMBO J.* **1992**, *5*, 1913–1919.

(8) (a) Goodchild, J.; Kohli, V. *Arch. Biochem. Biophys.* **1991**, *284*, 386–391. (b) McCall, M. J.; Hendry, P.; Jennings, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 5710–5714.

0002-7863/93/1515-8483$04.00/0 © 1993 American Chemical Society

linkers.[9] Use of linkers greatly simplifies the preparation of hammerhead-like catalysts composed of as few as 22 nucleosides. Four types of complexes were prepared. Sequences **1–5** (Figure 1) maintain an intact stem II but replace the four nucleotides of loop II with linkers based on hexaethylene glycol (**1**), bis(triethylene glycol) phosphate (**2**), tris(propanediol)bisphosphate (**3**), bis(propanediol)phosphate (**4**), and propanediol (**5**). The remaining complexes (Figure 1) contain similar linkers, but either the native four-base-pair stem (stem II) is reduced to two base pairs (**6**, **7**) or one base pair (**8–10**), or the stem is completely removed from the structure (**11–14**). The use of these derivatives permits the generation of a series of complexes that vary in the length of the synthetic linker (thus the spacing of the tethered nucleoside residues), and the presence or absence of one or more negatively charged phosphodiesters also varies with the linker used. The linker building blocks were prepared as (4,4′-dimethoxytrityl)-(β-cyanoethyl)phosphoramidite derivatives[9] and incorporated into the sequence at the desired site under the same conditions used for the coupling of nucleoside phosphoramidites. The linkers were incorporated into the sequences with yields that were comparable with those typically obtained for the four common nucleosides. Analysis of the sequences by polyacrylamide gel electrophoresis (PAGE) indicated the presence of a single species, and the mobility of the various RNA/linker sequences was directly related to the number of nucleoside residues present. The presence of additional phosphodiester residues in the linkers increased the distance of the migration.

Cleavage activity was measured under single turnover conditions with a large excess of ribozyme to ensure complexation of the substrate sequence. First-order rate constants characterizing the cleavage reaction were calculated from the half-lives of the complexes[10] and were corrected for the extent of cleavage at t_∞[11] (Table I, $\%P_\infty$). Substituion of the hexaethylene glycol (**1**) or bis(triethylene glycol)phosphate (**2**) linkers in place of the GUUA tetranucleotide loop of the native sequence results in activity that is very similar to that of the 34-mer ribozyme. As the linker replacing loop II (GUUA) is shortened to 16 atoms (**3**), 11 atoms (**4**), and 5 atoms (**5**), the cleavage rate decreases markedly. Previous work has indicated that a chain length of nine carbon atoms is required to bridge the terminal phosphates of an RNA duplex.[9b] While **3** and **4** provide this chain length, the presence of one or more negatively charged phosphodiesters may result in destabilizing repulsive electrostatic forces.

The number of base pairs in the stem can be reduced from four to two with the use of the neutral hexaethylene glycol linker without any change in cleavage rate (compare **6** with **1**), but the similar sequence **7**, with two negatively charged phosphodiesters, residues is less active. Two additional complexes containing the hexaethylene glycol linker, one with a single base pair (**8**), and one without any base pair (**11**), of stem II are also active catalysts, but the cleavage rates are reduced by 10- and 200-fold, respectively. The hexaethylene glycol linker is similar to a crown ether, and magnesium binding to the linker could assist in stabilizing the stem/loop II region.

The reduced cleavage rates with **8** and **11** suggest that the presence of a base pair or similar structure at the base of stem II is critical for formation of the active complex. Sequences comprised of a phosphodiester-containing linker and either a single C-G base pair (**9** or **10**) or no base pair (**12**, **13**, and **14**) in stem II are all less active than the corresponding complexes constructed with the neutral hexaethylene glycol linker. All of the linkers employed in complexes **8–14** are longer than the nine-carbon minimum, but the presence of additional charge at some sites may destabilize the active complex. Complex **13**, lacking any of the base pairs of stem II and containing a linker with two negatively charged phosphodiesters, did not exhibit any measurable cleavage activity.

Table I. Cleavage Rates for Ribozymes Containing Simple Linkers[a]

ribozyme	k_f (min^{-1})	relative cleavage rate	$\%P_\infty$[b]
native	1.1	1.0	88
1	0.81	0.74	89
2	0.71	0.65	91
3	0.12	0.11	86
4	0.0075	0.0068	82
5	0.0034	0.0031	81
6	1.2	1.1	87
7	0.0026	0.0024	69
8	0.11	0.10	81
9	0.050	0.045	75
10	0.032	0.029	80
11	0.0052	0.0047	71
12	0.0055	0.0050	71
13	nc[c]		
14	0.0060	0.0055	74

[a] Two 25-μL solutions containing either 1.2 μM ribo~me or 0.2 μM substrate in 50 mM Tris–HCl (pH 7.5) were heated to 95 °C for 1 min and cooled to 25 °C. Each solution of ribozyme was adjusted to 10 mM $MgCl_2$ and incubated at 25 °C for 15 min. The substrate solution was adjusted to 10 mM $MgCl_2$ prior to use. The reaction was inititated by mixing the two solutions (final ribozyme concentration = 0.6 μM, final substrate concentration = 0.1 μM). Aliquots of 5–7 μL were withdrawn, and the reaction was quenched by the addition of an equal volume of 50 mM Na_2EDTA/7 M urea/10% glycerol/0.05% xylene cyanol/0.05% bromphenol blue. The extents of cleavage were analyzed by PAGE on 20% gels. [b] $\%P_\infty$ = percent product at t_∞ ($t_\infty \geq 10t_{1/2}$, except **7**, t_∞ = 38 h, 8.5 $t_{1/2}$). [c] nc = no significant cleavage was observed after incubation for 18 h.

In summary, the neutral hexaethylene glycol linker can be employed to replace all of the nucleotides of loop II and two of the base pairs of stem II without any observable loss of cleavage activity. Some loss of activity is observed as three or all four of the base pairs of stem II are eliminated, but these complexes remain active RNA cleaving molecules. Based upon such studies, it should be feasible to further simplify the ribozyme complex using non-nucleoside components to generate active hammerhead-like RNA cleavage catalysts. These simpler catalysts may be amenable to detailed structural characterizations by NMR or other methods. Structural details have been largely unobtainable for the larger native hammerhead ribozyme complexes.

Acknowledgment. This work was supported in part by a grant from the National Science Foundation (MCB-9206528). L.W.M. thanks the American Cancer Society for a Faculty Research Award (FRA-384).

Supplementary Material Available: Experimental details and figures showing (S1) the ^{31}P NMR spectrum of the 1,3-propane diol linker, (S2) PAGE of the 14-linker-containing ribozyme sequences, and (S3) a typical autoradiogram used to monitor cleavage of the 12-mer substrate (7 pages). Ordering information is given on any current masthead page.

(9) Such linkers have been used previously in the construction of simple duplexes, see: (a) Durand, M.; Chevrie, K.; Chassignol, M.; Thuong, N. T.; Maurizot, J. C. *Nucleic Acids Res.* **1990**, *18*, 6353–6359. (b) Ma, M. Y.-X.; Reid, L. S.; Climie, S. C.; Lin, W. C.; Kuperman, R.; Sumner-Smith, M.; Barnett, R. W. *Biochemistry* **1993**, *32*, 1751–1758.

(10) $k_f = 0.693/t_{1/2}$.

(11) The determination of t_∞ was at a minimum of $10t_{1/2}$ [**7** was incubated for 38 h ($8.5t_{1/2}$), **13** exhibited no significant cleavage].