Hammerhead Minizymes with High Cleavage Activity: A Dimeric Structure as the Active Conformation of Minizymes

Serguei V. Amontov[†] and Kazunari Taira*[,†,‡]

Contribution from the National Institute of Bioscience and Human Technology, AIST, MITI, and Institute of Applied Biochemistry, University of Tsukuba, Tsukuba Science City 305, Japan

Received May 31, 1995[⊗]

Abstract: A number of minizymes with short oligonucleotide linkers (one to four bases) instead of stem-loop II were constructed. Only two of the minizymes examined had cleavage activity similar to that of the full-sized ribozyme. The minizymes with high activity formed dimeric structures with a common stem II. A significant contribution to stabilization of such a dimeric structure is probably made by nonstandard base pairing and a Mg^{2+} ion in both catalytic cores of the dimer. Interaction with the substrate also stabilized the dimeric structures. Thus, active minizymes can be designed with a self-complementary linker sequence with G and C on 5′ and 3′ sides, respectively, of the linker which induces the intermolecular interactions that lead to formation of active dimers.

Introduction

Hammerhead ribozymes can cleave any RNAs with high sequence specificity via Watson−Crick-type recognition at stems I and III (Figure 1a), provided that the target site contains the NUX triplet (N = G, A, C, or U; X = A, C, or U). The efficiency of cleavage depends on the combination of N and X.[1−6] In attempts to identify functional groups and to elucidate the role of the stem II region, various modifications and deletions have been made in the stem II region.[7−12] Complete deletion of the stem-loop II abolishes the catalytic activity.[8−12] Although it was concluded initially that replacement of stem-loop II with a tetranucleotide (with resultant formation of small ribozymes that were designated minizymes) affected hammerhead activity to only a moderate extent,[8] more recent findings indicate that this type of alteration has a significant effect on hammerhead activity.[9,12] In fact, activities of minizymes were found to be reduced by 2−3 orders of magnitude, a result that led to the suggestion that minizymes might not be suitable as gene-inactivating reagents.[12] Thus, conventional hammerhead ribozymes with a deleted stem II (minizymes) have been considered to be crippled structures and have attracted minimal interest because of their extremely low activity, as compared to that of the full-sized ribozyme.

Tuschl and Eckstein determined the minimal sequence requirements in the stem-loop II region for full activity of the hammerhead ribozyme.[9] Stems with two base pairs (bp's) had essentially unaltered catalytic activity, which was independent of the composition of the tetraloop, as long as the $G_{10.1}-C_{11.1}$ bp was maintained.[9] NMR analysis and probing of the hammerhead ribozyme structure with ribonucleases also confirmed the existence of the $G_{10.1}-C_{11.1}$ bp.[13,14] It has, thus, become widely accepted that a stem with two bp's that includes the $G_{10.1}-C_{11.1}$ bp, in combination with an additional loop, is the minimal structure that provides the necessary stability for a ribozyme with high catalytic efficiency.

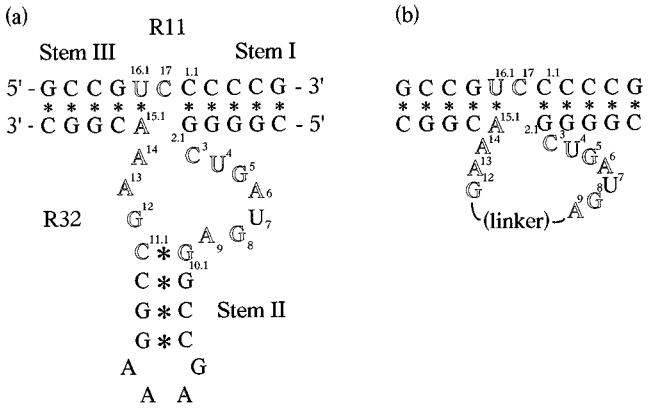

Figure 1. Secondary structure of (a) the hammerhead ribozyme (R32) and its substrate (R11) and (b) the complex of minizyme and substrate R11 used in this study. The length of the linker was varied between one and four nucleotides.

All previous investigations involved either complete deletion of the stem-loop II or maintenance of some base pairs, and no examination has yet been made of minizymes with mono-, di-, or trinucleotides that replace the stem-loop II. We now report that minizymes, designed with appropriate linker sequences, can form dimers with common stem II and satisfy the minimal sequence requirement (for bases $G_{10.1}$ and $C_{11.1}$) proposed by Tuschl and Eckstein, and these minizymes do indeed have high cleavage activity.

Materials and Methods

Synthesis of Ribozymes. Ribozymes and their corresponding substrate were synthesized on an ABI DNA/RNA synthesizer (model

[†] National Science Institute of Bioscience and Human Technology.
[‡] Institute of Applied Biochemistry.
[⊗] Abstract published in *Advance ACS Abstracts,* February 1, 1996.

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392; Applied Biosystems, Foster City, CA) and purified by HPLC and polyacrylamide gel electrophoresis as described previously.[15] Reagents for RNA synthesis were purchased either from American Bionetics, Inc. (ABN; Foster City, CA) or from ABI. Purification of the synthesized oligonucleotides was performed as described in the user bulletin from ABI (no. 53; 1989) with minor modifications.

Kinetic Measurements. Reaction rates were measured, in 25 mM $MgCl_2$ and 50 mM Tris-HCl (pH 8.0), under ribozyme-saturating (single-turnover) conditions at 37 °C. In all cases, kinetic measurements were made under conditions where all the available substrate was expected to form a Michaelis−Menten complex, with high concentrations of ribozyme (0.1−100 μM). The K_m value for the parent ribozyme (R32) at 37 °C under these conditions was 0.02 μM.[15]

Reactions were stopped by removal of aliquots from the reaction mixture at appropriate intervals and mixing them with an equivalent volume of a solution that contained 100 mM EDTA, 9 M urea, 0.1% xylene cyanol, and 0.1% bromophenol blue. Substrates and 5′-cleaved products were separated by electrophoresis on a 20% polyacrylamide/7 M urea denaturing gel and were detected by autoradiography. The extent of cleavage was determined by quantitation of radioactivity in the bands of substrate and product with a Bio-Image Analyzer (BA2000; Fuji Film, Tokyo). As mentioned above, all rates of reactions catalyzed by minizymes were determined under single-turnover conditions (with trace levels of 5′-^{32}P-labeled substrate), except in the case of the full-sized ribozyme in that the activity was determined from a multiple-turnover experiment.

Results and Discussion

Typical ribozyme sequences used in this study are shown in Figure 1. In order to ensure that we measure only the rate of the pure chemical cleavage step (k_{cleav}), all reactions in this study were carried out under single-turnover conditions.[15,16] Moreover, the rate-limiting nature of the cleavage step was confirmed for important constructs by checking the dependence on pH of their activities since Mg^{2+}-bound unprotonated oxygen plays a key catalytic role in reactions catalyzed by the hammerhead ribozyme.[17−19]

Rate constants for cleavage reactions are summarized in Table 1. Minizymes constructed in this study are designated by Mz and a number, which indicates the total number of nucleotides, together the composition of the linker (Figure 1b) that connects the conserved catalytic cores on the 5′ and 3′ sides. Mz22-GC, for example, is a minizyme of 22 nucleotides with the following sequence: 5′-CGGGG-CUGAUGA-(GC)-GAA-ACGGC-3′. The binding sites (stem I at the 5′-end and stem III at the 3′-end), consisting of 10 nucleotides in all, are underlined, and the components of the linker are shown in parentheses.

As can be seen from Table 1, most of the minizymes that we synthesized had very low cleavage activity. The weakly active minizymes included three out of four types of minizyme with a tetranucleotide linker, reflecting the findings of Long and Uhlenbeck.[12] Except in the case of Mz24-GCGC, the replacement of the stem-loop II by a tetranucleotide linker resulted in reduction of the cleavage rate by 3 orders of magnitude. To our surprise, Mz24-GCGC had high cleavage activity with a k_{cleav} of 2.2 min^{-1}, which was more than 50% of that of the full-sized ribozyme. The reduction in the rate caused by the GUAA sequence was 4−5 times greater than that by the UGAC and GTTC sequences. Introduction of trinucleotides similarly reduced the activity by 3 orders of magnitude: Mz23-UGA and Mz23-GCC had nearly the same activity as Mz24-UGAC and Mz24-GTTC.

Table 1. Activities of Various Ribozymes[a]

	k_{cleav} (min^{-1})	$K_{d(app)}$ (μM)	k_{rel} (%)
minizyme			
R32	4.0	0.10	100
Mz24-GCGC	2.2	0.17	55
Mz24-UGAC	0.006		0.15
Mz24-GTTC	0.007		0.18
Mz24-GUAA	0.002		0.05
Mz23-UGA	0.006		0.15
Mz23-GCC	0.008		0.20
Mz22−CG	0.0001		0.002
Mz22-GA	<0.0001		<0.002
Mz22-GC	2.5	5.1	65
Mz21-C	0.003		0.08
Mz21-G	<0.0001		
Mz21-U	<0.0001		
Mz21-A	<0.0001		
mixture of minizymes			
Mz24-UGAC/GTTC	0.12		3.0
Mz24-UGAC/GUAA	0.032		0.8
Mz21-C/Mz21-G	0.015	15	0.4
reported minizymes			
Mz-TTTT			0.12[b]
Mz-Linker			0.5[c]
Mz-UUUU			0.33[d]
Mz-UGAC			0.8[d]
Mz-CUCC			0.07[d]

[a] All reaction rates were measured, in 25 mM $MgCl_2$ and 50 mM Tris-HCl (pH 8.0), under ribozyme-saturating (single-turnover) conditions at 37 °C. In all cases, kinetic measurements were made under conditions where all the available substrate was expected to form a Michaelis−Menten complex, with high concentrations of ribozyme (0.1−100 μM). The K_m value for the parent ribozyme (R32) at 37 °C under these conditions was 0.02 μM.[15] [b] Taken from ref 9. [c] Taken from ref 11. [d] Taken from ref 12.

The most surprising finding was that, while minizymes with a dinucleotide linker, namely, Mz22-CG and Mz22-GA, were almost inactive (a reduction in rate of 4 orders of magnitude), Mz22-GC retained extremely high activity. Its activity was 65% of that of the parent ribozyme. We also examined whether the activity of minizymes with a mononucleotide linker, Mz21-N (N = C, G, U, or A), might exceed the activity of Mz22-GC. Among the four possible variants of the Mz21-N minizyme, only Mz21-C had the activity that was similar to that of the other minizymes with low activities (i.e., all minizymes except Mz22-GC and Mz24-GCGC). All other Mz21-N (N = G, U, or A) minizymes were found to be inactive.

When Mz22-GC (or Mz24-GCGC) is drawn as shown in Figure 1, it becomes clear that it is different in one importent respect from the full-sized hammerhead ribozyme. There is no Watson−Crick G−C base pair of the type proposed as the minimal requirement for the hammerhead catalytic center.[9] However, some of the kinetic features of the reactions catalyzed by Mz22-GC and MZ24-GCGC hinted at the formation of a dimer, in each case, as the catalytically competent molecule. Although Mz22-GC and MZ24-GCGC each had high activity under single-turnover conditions, they both had unexpectedly low activity under multiple-turnover conditions. Moreover, and in particular in the case of Mz22-GC, the $K_{d(app)}$ value determined under single-turnover conditions from a Lineweaver−Burk plot (Figure 2 and Table 1) was much higher than the K_m value for the complex of the full-sized ribozyme (R32) and its substrate despite the fact that both ribozymes had the identical substrate-binding site. Therefore, it seemed unlikely, that the $K_{d(app)}$ value reflected the dissociation constant of the complex of minizyme and its substrate. Thus, $K_{d(app)}$

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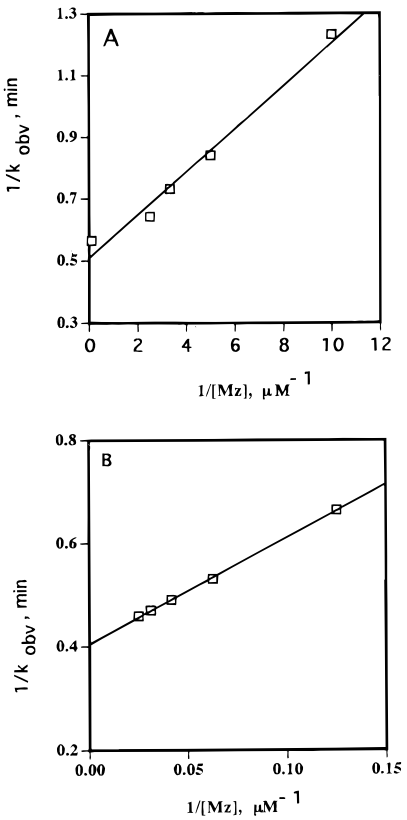

Figure 2. Lineweaver−Burk plots under single-turnover conditions for (A) Mz24-GCGC and (B) Mz22-GC. Calculated k_{cleav} and $K_{d(app)}$ values are 2.2 min^{-1} and 0.17 μM, respectively, for (A) Mz24-GCGC and the corresponding values are 2.5 min^{-1} and 5.1 μM for (B) Mz22-GC.

appears to be a complicated quantity, which might roughly characterize the dimerization process.

We tried to confirm the postulated dimerization of the minizyme (Figure 3) in a number of experiments by mixing different kinds of minizyme. Indeed, using pairs of minizymes with at least partially complementary linker sequences, we observed significant increases in cleavage activity after mixing, as compared with activities of the original individual minizymes. For example, as shown in Table 1, combinations of Mz24-UGAC and Mz24-GTTC, Mz24-UGAC and Mz24-GUAA, and Mz21-C and Mz21-G resulted in significantly higher cleavage activities than those of the respective individual minizymes (activities were increased by about 1 order of magnitude; Figure 4). These observation support our hypothesis, which is shown schematically in Figure 3.

In order to obtain more direct evidence that the unusually high cleavage activity of Mz22-GC and Mz24-GCGC can be accounted for by the formation of dimers, we synthesized two other minizymes. These minizymes had only "left" or "right" binding sites identical to the original binding site of the other hammerhead ribozymes (Figure 1) and were designated minizyme left (MzL) and minizyme right (MzR), respectively. These minizymes would only be able to form a binding site complementary to the conventional substrate sequence (R11 in Figure 1a) as a result of formation of such kind of dimer (Figure 5). The postulated heterodimer has two different binding sites. We also synthesized a noncleavable oligonucleotide with a sequence complementary to the second binding site, which is quite different from the conventional binding site. Investigation of the cleavage activity of this system and of each component of this system provided direct evidence that only a dimeric structure has reliable cleavage activity (Figure 6). Moreover, we found that, at relatively low concentrations of Mg^{2+} ions, the noncleavable oligonucleotide that was complementary to the second binding site (designated the pseudosubstrate) significantly enhanced the activity of the system, most probably via stabilization of the active dimeric structure (Figure 6). High concentrations of Mg^{2+} ions neutralized this effect (data not shown). It is likely, therefore, that Mg^{2+} ions can stabilize the dimeric structure independently by coordination with several groups in both catalytic cores. It remains to be determined whether the

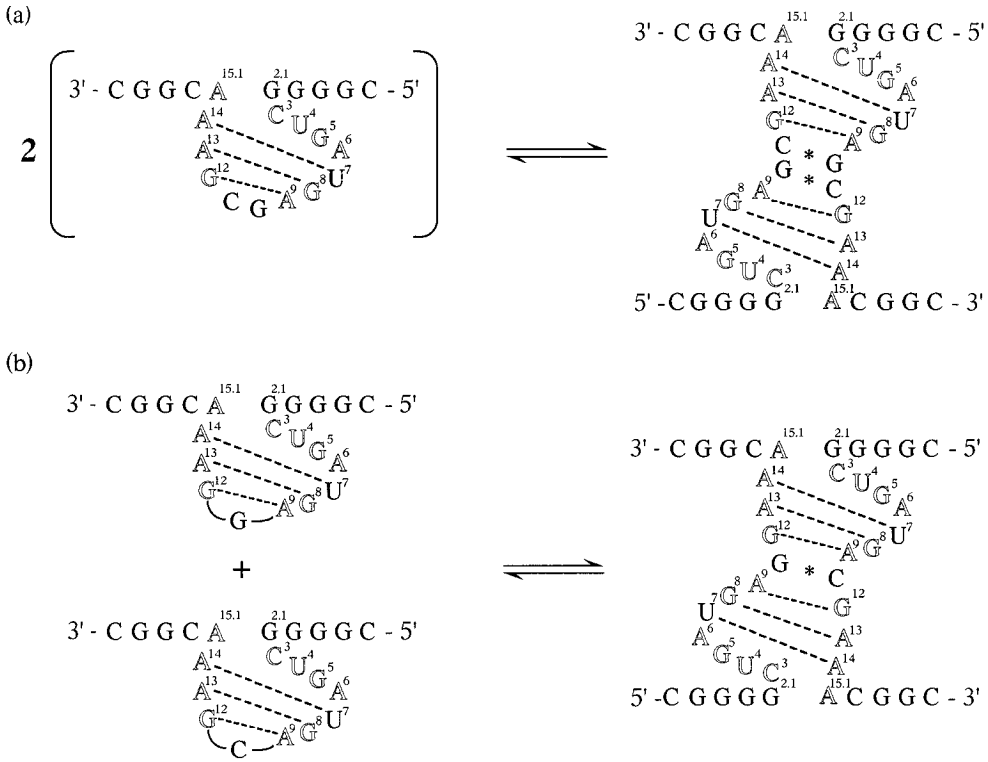

Figure 3. Dimerization of minizymes. (a) Homodimer of the Mz22-GC minizyme. (b) Heterodimer consisting of Mz21-G and Mz21-C. The heterodimer was more active than either of the individual minizymes (see Table 1).

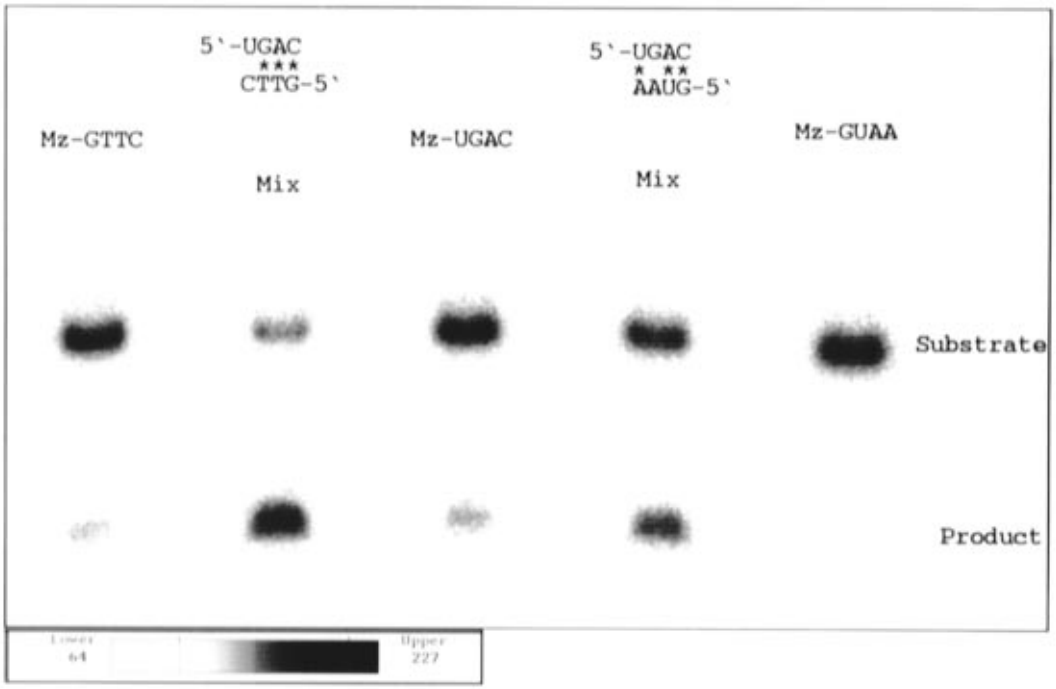

Figure 4. Cleavage activity of mixtures of minizymes with different oligonucleotide linkers. Concentrations: 5′-^{32}P-labeled substrate RNA, 20 nM; minizymes, total concentration of each species, 80 μM (lanes Mix, equimolar concentrations, namely, 40 μM, of each minizyme). When linkers of mixed minizymes were complementary to each other, heterodimers were probably formed and the cleavage rate increased. Minizymes used in this study are designated by the composition of the linker (Figure 1b) that connects the conserved catalytic cores on the 5′ and 3′ sides.

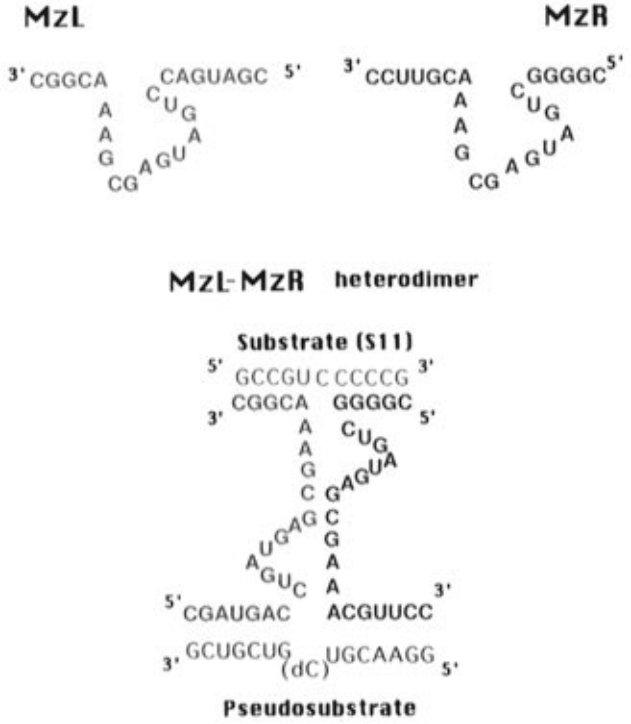

Figure 5. Schematic representation of the MzL−MzR heterodimer. Only after the formation of the heterodimer could the substrate (R11) used in this study (Figure 1) be cleaved. The MzL−MzR heterodimer can generate two different binding sites: one is complementary to the sequence of R11 (top), and the other is complementary to an uncleavable pseudosubstrate (bottom). The presence of the pseudosubstrate stabilized the dimer, thereby enhancing the cleavage of R11 (see Figure 6).

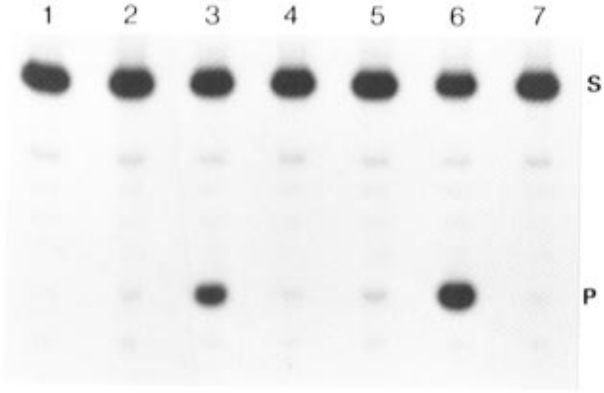

Figure 6. Cleavage activity of the MzL−MzR heterodimer. Bands in each lane represent the reaction product formed in 4 min at 37 °C in 50 mM Tris-HCl (pH 8.0) and 25 mM $MgCl_2$. Concentrations: 5′-^{32}P-labeled substrate RNA (S), 0.2 μM; MzL, 2 μM; MzR, 1 μM; pseudosubstrate (PsS), 2.5 μM. Lanes: 1, S; 2, S + MzL; 3, S + MzL +MzR; 4, S + MzR; 5, S + MzL + PsS; 6, S + MzL + MzR + PsS; 7, S + MzR + PsS P = product. When a more stable MzL−MzR heterodimer was formed in the presence of the pseudosubstrate (lane 6), the cleavage activity was highest. Unless there was a possibility for formation of the MzL−MzR heterodimer (lanes 3 and 6), no cleavage activity of the individual minizymes was detected.

Mg^{2+} ion that establishes the active form of ribozyme is the same as the catalytically indispensable Mg^{2+} ion.[17−20]

The recently reported three-dimensional structure of a hammerhead ribozyme indicates that the three nucleotides U_7, G_8, and A_9 pair with G_{12}, A_{13}, and A_{14} to form a mismatched duplex in the central catalytic loop.[21,22] It is likely that a similar mismatched duplex contributes to the stabilization of the mixed dimer composed of, for example, Mz21-C and Mz21-G (Figure 3b). A single C−G pair formed by a mononucleotide linker is unlikely to stabilize the dimer sufficiently. It should also be noted that the $K_{d(app)}$ value decreased with increases in the number of base pairs in the linker region of each minizyme (compare the $K_{d(app)}$ of 5.1 μM for Mz22-GC with the $K_{d(app)}$ of

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0.17 μM for Mz24-GCGC). This result supports the earlier conclusion that the $K_{d(app)}$ represents the dissociation of dimers.

Dimeric hammerheads have already been described.[23] In the reported case, the hammerhead monomer had low activity because of a rudimentary stem III and only dimerization yielded a common but adequate stem III, with relatively high resultant self-cleavage activity. In the present work we discovered a special case, wherein a monomeric hammerhead minizyme is almost completely inactive as a cosequence of the absence of stem II and, thus, has an incomplete hammerhead catalytic center. Only dimerization allows reconstruction not only of a common stem II but also of a perfect catalytic center.

This type of dimerization might explain some phenomena observed in previous studies.[8,10,11] For example, Fu et al. found that hammerhead ribozymes with non-nucleoside linkers, instead of oligonucleotide hairpin loop II, and with a reduced stem II (two base pairs) require a $G_{10.1}-C_{11.1}$ and $C_{10.2}-G_{11.2}$ stem sequence. An alternative $G_{10.1}-C_{11.1}$ and $G_{10.2}-C_{11.2}$ stem sequence resulted in inactive ribozymes (under single-turnover conditions).[11] Fu et al. considered this restriction to be an additional common requirement in cases of a short (two-bp) stem II. At that time it was difficult to explain this phenomenon, but now it may be possible to explain it in the context of dimerization. As a result of the special features of the ribozyme sequence used in the earlier studies, ribozymes with non-nucleoside linkers and a $G_{10.1}-C_{11.1}$, $G_{10.2}-C_{11.2}$ stem sequence might form (at least under single-turnover conditions) stable inactive dimers (Figure 7a). By contrast, when there is a $G_{10.1}-C_{11.1}$, $C_{10.2}-G_{11.2}$ stem sequence, a normal active dimeric structure (Figure 7c) seems more stable than the inactive counterpart (Figure 7d). Thus, in stem II-shortened constructs, it is necessary to check whether the non-nucleoside linkers really form a structure analogous to a hairpin loop (as shown in Figure 7b) or whether there is a possibility of dimerization (Figure 7c). Some other observations with stem II-shortened constructs can also be tentatively explained by a dimerization process.

It is to be emphasized that not all minizymes form dimeric structures.[8,24] In general, monomeric minizymes are significantly less active than the comparable full-sized ribozymes when cleaving short substrates. However, Hendry et al. recently demonstrated that particular monomeric minizymes were more active than full-sized ribozymes when a long transcribed RNA was used as a substrate.[24]

In conclusion, minizymes with appropriate linker sequences can dimerize to form catalytically active structures that satisfy the minimal sequence requirements for hammerhead ribozymes. Minizymes can be highly active when they have a self-complementary linker sequence with G and C on the 5′ and 3′ sides, respectively, of the linker that induce intermolecular interactions via formation of a dimer, as shown in Figure 3. The stability of dimeric structure depends on the number of base pairs in stem II of the dimer. It is also likely that a significant contribution to stabilization of dimers is made by additional non-Watson−Crick base pairing and coordination of Mg^{2+} ions within the catalytic core. Interactions with the substrate can also stabilize the dimeric struture.

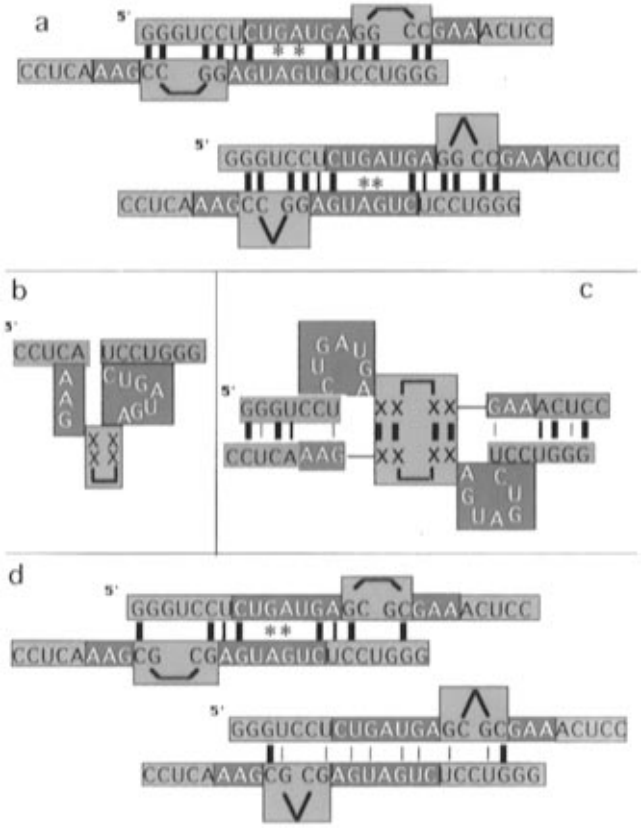

Figure 7. Possible structures of previously reported ribozymes with non-nucleotide linkers:[11] a and d, variants of an inactive dimer with $G_{10.1}-C_{11.1}$, $G_{10.2}-C_{11.2}$ and $G_{10.1}-C_{11.1}$, $C_{10.2}-G_{11.2}$ stem II, respectively; b, monomer structure proposed previously;[11] c, active dimer. Stem II regions with non-nucleotide linkers are boxed in blue (non-nucleotide linkers are indicated by solid lines within the blue box). Stronger hydrogen-bonding interactions are indicated by thicker lines. A*G base pairing may provide an additional stabilization of the duplex. The catalytic site and the binding arms are indicated by red and green regions, respectively.

Acknowledgment. The authors wish to thank Dr. Philip Hendry at CSIRO, Sydney, Australia, for his very important comments and for providing us with his experimental results prior to publication[24] and to Drs. P. K. R. Kumar, T. Shimayama, and S. Sawata for helpful discussions.

JA9517734

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