# Enhanced Folding of Hairpin Ribozymes with Replaced Domains†

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*Received March 13, 1996; Revised Manuscript Received May 14, 1996*⊗

ABSTRACT: Reversely joined ribozymes (Komatsu et al., 1995) have been proven to be active. Here we describe the construction of hairpin ribozymes with separated domains, but containing complementary arms for association of the two domains. Linker nucleotides were inserted between the arms and domains. These ribozymes were active under the standard conditions (12 mM $MgCl_2$), depending on the length of the linker. When the complementary arms were covalently joined through a stable loop, these ribozymes showed cleavage activities. However, the $K_m$ value of the stem−loop ribozymes was found to be larger than that of the parent ribozyme, which can adopt both linear and bent conformations. Kinetic analyses of these modified hairpin ribozymes suggest a higher turnover of the hairpin ribozyme as compared to other small ribozymes. The present ribozymes provide insight into the nature of the domain interaction and are suitable for physicochemical studies on the tertiary structure of the hairpin ribozyme.

Small catalytic RNAs, discovered in various types of viroid, virusoid, and virus satellite RNAs (Symons, 1992), are known to act as enzymes for RNA cleavage or ligation reactions. The sequences required for the catalytic activities have been characterized and named hammerhead (Forster & Symons, 1987) or hairpin (Hampel & Triz, 1989) depending on their secondary structures. Several ribozymes have been used to cleave target RNA in cultured cells (Sarver et al., 1990; Koizumi et al., 1992; Bratty et al., 1993; Christoffersen & Marr, 1995). In particular, hairpin ribozyme has been used as an effective gene therapeutic agent because of its high cleavage activity (Yu et al., 1993; Leavitt et al., 1994). The hairpin ribozyme derived from the catalytic center of the negative strand of the satellite RNA of tobacco ringspot virus (sTRSV) is responsible for the cleavage and ligation reactions of the satellite RNA during replication (Buzayan et al., 1986; Haseloff & Gerlach, 1989; Feldstein et al., 1989). This ribozyme contains 50 bases and can cleave RNA that binds to the ribozyme by base-pairing (Hampel & Tritz, 1989; Hampel et al., 1990). The cleavage reaction occurs by an in-line mechanism, requires the presence of magnesium ions (Van Tol et al., 1990), and yields a 2′,3′-cyclic phosphate and 5′-hydroxyl group. It has been suggested that the hairpin ribozyme cleaves RNA by a different mechanism from that of the hammerhead ribozyme with respect to the requirement of metal ions (Dahm & Uhlenbeck, 1991; Chowrira et al., 1993), from the results of phosphorothioate substitution experiments (Buzayan et al., 1988; Koizumi & Ohtsuka, 1991; Chowrira & Burke, 1992). The hairpin ribozyme contains four stem regions (helices 1−4) and two internal loops in domains I and II (Figure 1a; Hampel et al., 1990; Berzal-Herranz et al., 1993). Essential bases required for the cleavage activity in the internal loops have been identified by an in vitro selection method (Berzal-Herranz et al., 1992, 1993; Joseph et al., 1993). Point mutations (Sekiguchi et al., 1991; Anderson et al., 1994; Grasby et al., 1995), chemical modification (Butcher & Burke, 1994b), and UV cross-linking analyses (Butcher & Burke, 1994a; dos Santos et al., 1993a,b) of the ribozyme have provided further information about the secondary structure and the required sequences. These experiments

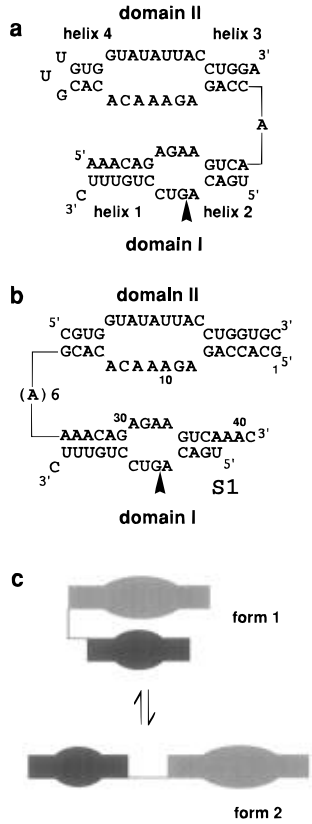


FIGURE 1: Sequences of hairpin ribozymes. (a) Postulated secondary structure of the catalytic domain of the sTobRV(−) hairpin ribozyme. Domain I consists of helix 1, helix 2, and the internal loop, and domain II consists of helix 3, helix 4, and the large internal loop. (b) Reversely joined ribozyme with a six-adenosine linker. (c) Schematic drawing of the relative positions of the domains of a hairpin ribozyme. Form 1 indicates the active bent form and form 2 the inactive extended form. Domain I and domain II are drawn in black and gray, respectively. Arrows indicate the cleavage sites.

† This work was supported by Special Coordination Funds for Promoting Science and Technology of the Science and Technology Agency of the Japanese Government, and by a grant-in-aid from the Ministry of Education, Science and Culture of Japan.

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⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1996.

S0006-2960(96)00627-7 CCC: $12.00 

suggest that unusual base pairs, similar to the one found in the hammerhead ribozyme, exist in domain II. The 2-amino group of guanosine at the 3′-cleavage site is essential for the cleavage reaction (Chowrira et al., 1991; Grasby et al., 1995), and this feature is unique and is different from other small catalytic RNAs. There are similar sequences in the satellite RNAs of chicory yellow mottle virus (sCYMV; Rubino et al., 1990) and arabis mosaic virus (sArMV; Haseloff & Gerlach, 1989), and they also have RNA cleavage activities (DeYoung et al., 1995); however, they cleave NGUA most effectively, whereas NGUC is cleaved fastest by the hairpin ribozyme derived from sTRSV.

The three-dimensional structure of the hammerhead ribozyme has been demonstrated by X-ray analysis (Pley et al., 1994; Scott et al., 1995). However, the ternary structure of the hairpin ribozyme has not been clarified. Since the internal loop that is distantly located from the cleavage site is also essential, a multifolded structure may be the active conformation for the cleavage activity. Linker insertion experiments suggested that the active conformation contains a bent form (Feldstein & Bruening, 1993; Komatsu et al., 1994). We have also shown that a new hairpin ribozyme, in which two domains were replaced, was still active (Komatsu et al., 1995). From these results, it is clear that the domain−domain interaction leads to the cleavage reaction, and a bent form is essential for the RNA cleavage activity. Recently, Burke and co-workers showed that the completely separated domains of the hairpin ribozyme were active under high ribozyme and magnesium concentrations (Butcher et al., 1995). Their results support the idea that a bend exists in the catalytic topology of the hairpin ribozyme. Since the active conformation of the ribozyme presumably requires an unknown domain−domain interaction, it may be difficult for the completely separated domains to maintain the associated form in low magnesium concentrations.

A ribozyme with two separately engineered domains connected by six adenylate linkers was more active than that with cytidylates (Figure 1b, Komatsu et al., 1995). In this reversely joined ribozyme, the active bent conformation is maintained only by the internal loop−loop interaction. There may be an alternative, inactive conformer, in which the relative position of two domains is extended. Therefore, it is thought that there is an equilibrium between the bent and the extended conformations in the rejoined ribozyme (Figure 1c). This inactive conformation would disrupt a structure analysis by nuclear magnetic resonance (NMR)[1] or X-ray methods. In this paper, we describe separated hairpin ribozymes with complementary arms that assist to form the active bent conformation (Figure 2a). We also prepared joined ribozymes with a stable hairpin loop. The activities of these ribozymes were compared with that of the parent reversely joined ribozyme. The ribozymes designed here suggest a possible mechanism of higher turnover numbers of the hairpin ribozyme as compared to the hammerhead ribozyme. These stabilized ribozymes seem to be suitable for X-ray or NMR analyses of the tertiary structure of hairpin ribozymes.

## MATERIALS AND METHODS

*RNA Synthesis.* All RNAs were synthesized using phosphoroamidite units purchased from GLEN Research. The synthesis was carried out with an Applied Biosystems DNA/RNA synthesizer (model 394A). All RNAs were synthesized with dimethoxytrityl groups at their 5′-ends. Deprotection of the bases was performed in c. $NH_4OH$/EtOH (3:1, v/v) at 55 °C for 16 h. The 2′-TBDMS groups were removed by 1.0 M tetrabutylammonium fluoride (TBAF, Aldrich) in THF at 37 °C for 18 h (Scaringe et al., 1990), and the reaction was quenched by 5 volumes of 0.1 M triethylammonium acetate buffer (pH 7.0). After purification on an open C-18 column preequilibrated with 50 mM triethylammonium bicarbonate (pH 7.5), the dimethoxytrityl group was removed in hydrochloric acid (pH 2) for 15 min at room temperature. The reaction was neutralized with c. $NH_4OH$ and desalted on Sephadex G25. RNAs were purified by reverse phase and anion exchange column chromatographies, as described previously (Komatsu et al., 1993), using $\mu$-Bondasphere (C-18), 3.9 mm i.d. x 150 mm (Waters), and TSK gel DEAE-2SW, 4.6 mm i.d. x 250 mm (Tosoh), columns, respectively. Substrate RNAs were 5′-labeled according to the previous report (Sekiguchi et al., 1991).

*Cleavage Reactions of Ribozymes with Separated Domains.* The ribozyme solutions contained equivalent moles of domain I and domain II. The ribozyme (4 $\mu$M) and the substrate solutions (40 nM) were dissolved separately in the cleavage buffer (40 mM Tris-HCl, 12 mM $MgCl_2$, and 2 mM spermidine-3HCl). The ribozyme (30 $\mu$L) and the substrate solutions were heated at 90 °C for 2 min and immediately transferred to an ice bath, and aliquots were preincubated separately at 32 °C for 7 min. After the preincubation, an equal volume of the ribozyme was added to the substrate solution to start the reaction. The reaction mixture was incubated at 32 °C, and aliquots were taken at time intervals and added to loading solution containing 8 M urea and 50 mM EDTA to stop the cleavage reaction. The percentages of the cleavage were determined by a Bioimaging analyzer (FUJIX BAS2000). Half-lives for substrate cleavage ($t_{1/2}$) were determined from a least squares fit of the data points on a plot of the logarithm of the percentage of the remaining substrate versus time. Cleavage rate constants were obtained from the relationship $k = 0.693/t_{1/2}$.

*Cleavage Reactions with the Hairpin Ribozymes Containing a Stable Loop.* The ribozyme strands of the three complexes and the substrate were dissolved separately in the cleavage buffer, and were denatured by heating at 65 °C for 2 min, followed by cooling in an ice bath. After the ribozyme and substrate solutions were preincubated separately at 37 °C for 7 min, an equal volume of ribozyme solution was added to the substrate solution, and the reaction was started in a final volume of 20 $\mu$L, containing 5−50 nM ribozyme strands and 0.02−1.5 $\mu$M substrate. From the cleavage products, the initial velocities were calculated over a time range that was within a specified range (<10% cleavage), and the kinetic parameters were obtained from Hanes−Woolf plots. The first-order rate constants ($k_{cl}$) were determined using 20 nM 5′-end-labeled substrate treated with various concentrations of ribozyme strands to saturate the substrate at 37 °C.

*Gel Mobility Shift Assay.* The ribozyme strands of the three complexes and the substrate (SdA, 40 nM) were dissolved separately in cleavage buffer containing 3% glycerol. After the ribozyme and substrate solutions were heated at 90 °C for 2 min and immediately transferred to an ice bath, each solution was incubated at 37 °C for 7 min, an

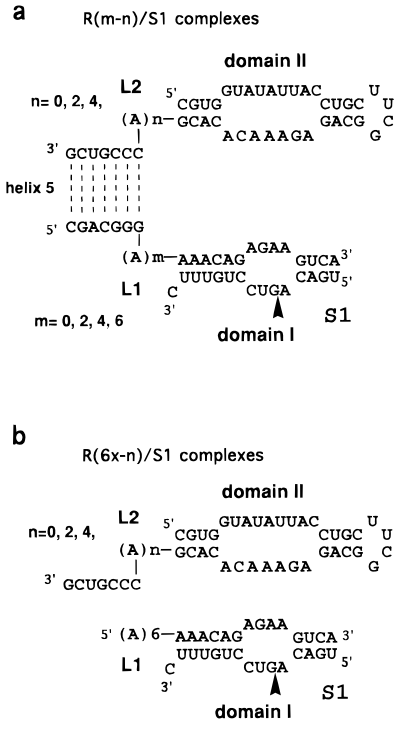
[1] Abbreviations: S and R, substrate and ribozyme, respectively; NMR, nuclear magnetic resonance; TBDMS, *tert*-butyldimethylsilyl; TBAF, tetrabutylammonium fluoride.

equal volume of ribozyme solution was mixed with the substrate, and then the mixture was incubated at 37 °C in a final volume of 10 $\mu$L. Thirteen concentrations of the ribozyme strands were studied from 5 nM to 500 nM. After an hour of incubation, the gel mobility shift assay was performed using a 10% [acrylamide: bis(acrylamide) ratio of 29:1] native polyacrylamide gel in 40 mM Tris−acetate (pH 7.5) and 12 mM magnesium acetate buffer at room temperature. The percentages of complex formation were measured by a BAS 2000 (FUJIFIX). The dissociation constants ($K_d$) were calculated from the plots of the percentage of the complex versus the ribozyme concentration, using the equation $K_d$ = [ribozyme; total at midpoint] − $^1/_2$[SdA; total] (Pyle et al., 1990).

The conformational analysis of the three stem−loop ribozyme complexes was performed by a similar procedure as that used for the $K_d$ measurement. The uncleavable substrate analog (SdA) was combined with the ribozyme strands. The aliquots contained 20 nM SdA and 1 $\mu$M ribozyme strands in the cleavage buffer (40 mM Tris-HCl, 12 mM $MgCl_2$, and 2 mM spermidine-3HCl; 10 $\mu$L). After the solution was incubated for 1 h, 30% glycerol was added to achieve a final 6% glycerol concentration. The complex formation was analyzed using an 8% (acrylamide:bis-(acrylamide) ratio of 29:1] nondenaturing polyacrylamide gel in 40 mM Tris−acetate (pH 7.5) and 12 mM magnesium acetate buffer at 4 °C.

## RESULTS AND DISCUSSION

*Divided Hairpin Ribozymes with Complementary Arms.* Divided hairpin ribozymes with complementary arms (helix 5) and adenylate linkers were constructed as shown in Figure 2a. In these ribozymes, the two domains are separated, but they are able to associate by the complementary base-pairing arm that can play a role as an anchor for the noncovalent interaction of the two domains to maintain an active, bent form (form 1, Figure 1c). The adenosine linkers (L1, $m$ = 0−6; L2, $n$ = 0−4) were inserted between the two domains and the base-pairing arms. Twelve complexes were constructed by combinations of domain I and domain II (Figure 2). These ribozymes are named R(L1−L2), in which L1 or L2 refers to the number of adenylate residues in the linker. Separated domains without a complementary strand [R(6$X$−$n$), Figure 2b] were prepared as control molecules. Since the binding of the two domains is essential to evaluate the effect of the linker lengths on the cleavage activities, the cleavage reactions were carried out under single-turnover conditions, and the first-order rate constants were calculated (Ruffner & Uhlenbeck, 1990). The analysis of the cleavage reactions indicated that the complexes, except for R(0−0) (without either L1 or L2 linker), had cleavage activities (Figure 3). Psuedo-first-order rate constants were determined, and are shown in Figure 4. The values are shown in Table 1 together with the results of the control molecules. The rates of the cleavage reactions became higher as either the L1 or the L2 linker length increased, and particularly seemed to depend on the length of L1. Although R(4−2) and R(4−4) had about 80-fold higher activities as compared to those for R(0−2) and R(0−4), respectively, R(2−4) and R(4−4) showed 10-fold activity over R(2−0) and R(4−0). When the number of bases in L1 was changed from 4 to 6, the cleavage activities increased about 2-fold. The ribozymes with the same L1 linkers [R(6−2) and R(6−4), R(4−2) and R(4-4), R(2−2) and R(2−4)] had almost the same activities.




| R(m-n) | m | n |
|---|---|---|
| R(0-0) | 0 | 0 |
| R(0-2) | 0 | 2 |
| R(0-4) | 0 | 4 |
| R(2-0) | 2 | 0 |
| R(2-2) | 2 | 2 |
| R(2-4) | 2 | 4 |
| R(4-0) | 4 | 0 |
| R(4-2) | 4 | 2 |
| R(4-4) | 4 | 4 |
| R(6-0) | 6 | 0 |
| R(6-2) | 6 | 2 |
| R(6-4) | 6 | 4 |

FIGURE 2: Separated domain hairpin ribozymes. (a) R($m$−$n$)/S1 complexes. Ribozymes contain both the substrate binding region and domain II. Domain I and domain II involve L1 ($m$) and L2 ($n$) linkers, respectively, and R($m$ − $n$) indicate ribozyme complexes ($m$ = 0, 2, 4, 6; $n$ = 0, 2, 4). (b) Control molecules; R(6$X$−$n$)/S1 complexes lack the hybridizing arms of domain I. Solid lines and dotted lines show covalent and hydrogen bonds, respectively.

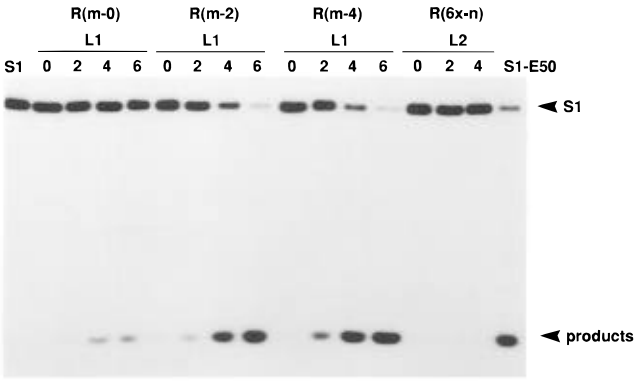



FIGURE 3: Autoradiogram of 20% denaturing polyacrylamide gel analysis of the reactions with separated domain ribozymes. The number of linkers (L1 or L2) is indicated at the top of the gel. 6$X$ in the L1 row indicates domain I without hybridizing arms. E50 is a wild-type hairpin ribozyme (Figure 1a). The 5′-end-labeled substrates (20 nM) were treated with the ribozyme R(L1−L2) (810 nM) for 20 min and E50 (20 nM) for 10 min at 32 °C.

These results suggest that the length of L1 influences the cleavage activity more than that of L2. However, the lack of L2 seemed to be disadvantageous for the cleavage. These differences in the contributions of L1 and L2 in the cleavage activities might be derived from their conformations. Although the above result suggests that the duplex formation of helix 5 is responsible for the cleavage activity, the control molecules shown in Figure 2b also were tested for their cleavage abilities. As shown in Table 1, the molecules without the hybridization arm [R(6$X$−$n$), $n$ = 0, 2, or 4] revealed little cleavage activity under the standard conditions. R(6−2) and R(6−4) had about 300 times higher activity than R(6$X$−2) and R(6$X$−4). These results confirmed that the high cleavage activities of R($m$−$n$) ($m$ = 2, 4, 6; $n$ = 2, 4)

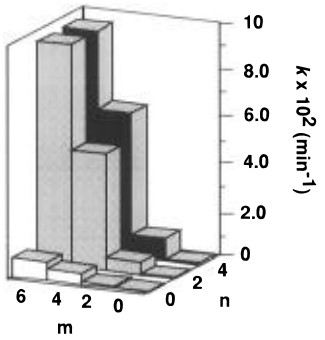



FIGURE 4: Cleavage rate constants versus L1 and L2 linker lengths. R($n$−0), white bars; R($n$−2), gray bars; R($n$−4), black bars ($n$ = 0, 2, 4, 6).

Table 1: Cleavage Rate Constants ($k \times 10^2$ min$^{-1}$) of Hairpin Ribozymes with Separated Domains[a]

| | L1, $m$ = | | | | |
|---|---|---|---|---|---|
| L2, $n$ = | 0 | 2 | 4 | 6 | 6*X* |
| 0 | 0.048 | 0.11 | 0.48 | 0.79 | 0.029 |
| 2 | 0.059 | 0.53 | 5.0 | 9.5 | 0.035 |
| 4 | 0.088 | 0.95 | 6.2 | 9.5 | 0.038 |

[a] The letters $m$ and $n$ indicate the number of adenine bases of the L1 and L2 linkers, respectively. Substrates (20 nM) were treated with 810 nM ribozyme strands at 32 °C in the presence of 12 mN $MgCl_2$.

resulted from the formation of helix 5. Recently, it was shown that the cleavage reaction occurred even in completely separated domains of the wild type of hairpin ribozyme (Butcher et al., 1995). However, the reactions were performed with high magnesium ion concentrations. The ribozymes with separated domains presented here show cleavage activities even under the standard buffer conditions (12 mM $MgCl_2$), because the association of the two domains was aided by the hybridizing arms of the two domains. Previously, we showed that longer linkers between the two domains were required for the high cleavage activity of reversely joined ribozymes (Komatsu et al., 1995). In the present experiments, linear linkers with the same lengths were not equal in their abilities to stimulate the cleavage. This showed that not only the linker length but also the position is important for the bent conformation fixed by the helix. These linkers seem to extend in the same direction as the helix. When the length of L2 is longer than L1, domain II is distant from both helix 5 and domain I. The interaction between domain I and domain II then seems to be disturbed. On the other hand, if the length of L1 is longer, domain I is close to domain II, with a favorable interaction. However, L2 must exist for efficient cleavage. If there was no L2 linker in domain II, helix 4 and helix 5 would become coaxial in the two-domain complex. These conformations should be stable from the point of the base-stacking effect. However, these coaxial conformations might be unfavorable for the domain−domain interaction, because there is no structural relaxation between the two domains to allow domain I to approach domain II.

*Two Hairpin Ribozymes Containing a Stable Loop.* In the ribozymes R($m$−$n$) examined above, the two domains are associated by only seven base pairs in helix 5, and the number of base pairs seems too low for the enzyme to turn over efficiently at 37 °C. For further stabilization of the bent conformation, ribozymes with a stable hairpin loop (Heus & Pardi, 1991) introduced at helix 5 were constructed

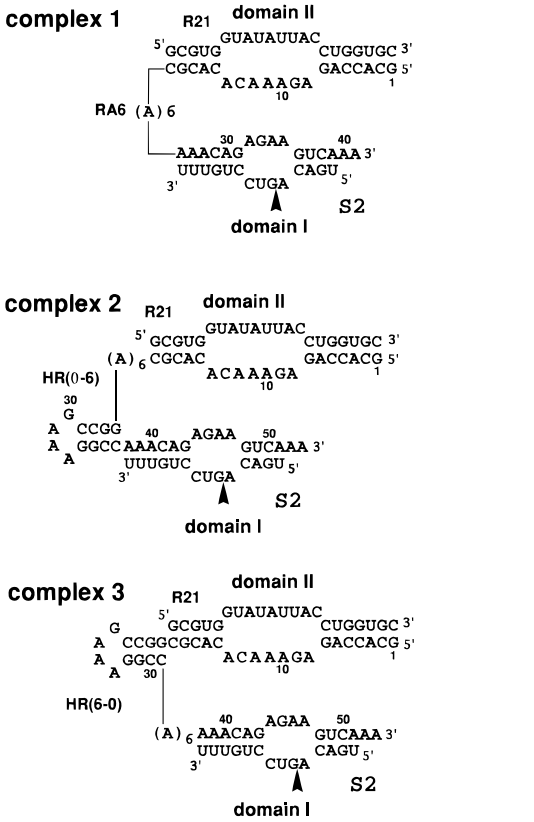



FIGURE 5: Secondary structures of the stemmed hairpin ribozyme−substrate complexes. Complex 1 consist of a reversely joined ribozyme with six adenine linkers and the S2 substrate. Complex 2 consists of HR(0−6), R21, and S2. Complex 3 consists of HR-(6−0), R21, and S2. Arrows and solid lines indicate the cleavage sites and the covalent bonds, respectively.

Table 2: Kinetic and Binding Parameters for Complexes 1−3 (Figure 5)

| | complex 1 | complex 2 | complex 3 |
|---|---|---|---|
| $k_{cat}$ (min$^{-1}$) | $4.7 \times 10^{-2}$ | ND | $3.5 \times 10^{-2}$ |
| $K_m$ (nM) | 22 | ND | 150 |
| $k_{cat}/K_m$ (min$^{-1}$·nM$^{-1}$) | $2.1 \times 10^{-3}$ | ND | $0.23 \times 10^{-3}$ |
| $k_{cl}$ (min$^{-1}$) | $1.2 \times 10^{-2}$ | $0.077 \times 10^{-2}$ | $2.4 \times 10^{-2}$ |
| $K_d$ (nM) | 9.5 | 8.0 | 10 |

(Figure 5). When helix 5 was coaxial with either helix 1 or helix 4, the cleavage activities were decreased in a range for the R($m$−0) or R(0−$n$) ribozymes, and the reason for the inhibition was unclear. To investigate the differences of the cleavage activities caused by the differences in the position of the inserted linker, two ribozymes with six adenosine linkers at L2 (complex 2) or L1 (complex 3) were prepared. These ribozymes formed complexes with substrate S2 (lacking the 3′-end cytidine of S1). The activities of these ribozymes were compared with that of complex 1, which had a linear linker instead of helix 5. The cleavage reactions were performed at 37 °C under multiple turnover conditions for measurement of the kinetic parameters (Table 2). Complex 3, which had only the L1 linker, showed almost the same $k_{cat}$ value with complex 1, although the $K_m$ of complex 3 was 7-fold larger than that of complex 1. Complex 2, with the L2 linker, did not work catalytically, and kinetic parameters were not obtained. In the active ribozyme (complex 3), it is likely that the substrate binding region of domain I (A38−A53) approached domain II and interfered with the binding to the substrate, because of the presence of the bent conformation aided by helix 5. To

investigate the cleavage abilities of these complexes, a labeled substrate was treated with excess amounts of the ribozyme to saturation concentrations, and the first-order rate constants ($k_{cl}$) were obtained. The values of $k_{cl}$ for complex 1 and complex 3 were almost the same as the $k_{cat}$, and complex 2 showed weak activity (Table 2). The two domains of complex 3 interact with each other more easily, due to the bent conformation, and the cleavage step seemed to be stimulated. The inactivity of complex 2 is consistent with the results obtained in the reaction of the separate domain ribozyme [R($m-n$)], and this confirms that the L1 linker in these ribozymes influenced the cleavage activity more than L2, even though the length of the linkers inserted between the two domains is the same in the cases of complex 2 and complex 3. These results also suggest that the coaxial orientation of helix 5 with helix 1 is responsible for the inactivity of the ribozyme, but that of helix 5 with helix 4 is not. Another reason for the inactivity of complex 2 may be derived from the base−base interactions of the adenosine linker with the substrate binding region in complex 2 (A38−A53), which might inhibit substrate binding to the ribozyme. To investigate whether the ribozyme strands of the three complexes bind to the substrate RNA, a gel shift analysis was performed. An uncleaved substrate (SdA; 5′UGACd-AGUCCUGUUU3′), in which 5A was replaced with deoxyadenosine, was synthesized, 5′-labeled, and mixed with the ribozyme strands at concentrations from 10 nM to 500 nM, in the presence of magnesium ions. It was proven that the three ribozymes formed complexes with the substrate analog under the conditions of the gel shift analysis, and the $K_d$ values for these complexes were obtained (Table 2). Since the $K_d$ values for the three complexes were almost the same, the formation of complex 2 was also confirmed. This result indicates other possibilities for the inactivity of complex 2. This difference in the activities between complex 2 and complex 3 may result from a difference in the conformations between the two complexes, as discussed in the case of the separated domain ribozymes [R($m-n$), Figure 2].

*Conformational Analysis of the Hairpin Ribozyme by Insertion of a Single Linker*. Native gel electrophoresis was carried out to compare the conformations of both complex 2 and complex 3, which were different in the linker position. The complex formations were analyzed by using the 5′-end-labeled substrate (SdA) and each ribozyme strand [R21, RA6, HR(6−0), or HR(0−6)]. The substrate analog did not bind with R21 (the 5′ side fragment of the ribozyme) (lane 2, Figure 6). However, the 3′-side fragments [HR(0−6), HR-(6−0), or RA6] of the ribozymes were able to bind to SdA, because these 3′-side fragments have the substrate binding region (lane 3, lane 4, and lane 5). Three complexes were found to be formed by combining SdA with the ribozyme strands. Complex 1, with the smallest molecular weight, moved fastest in the gel. However, complex 2 was retarded more than complex 3, which has the same molecular weight as complex 2. This difference in the mobility suggested a difference in their conformations. Since similar differences in migrations were also detected in SdA mixed with the 3′-side ribozyme fragment, the conformations of the SdA−3′-side ribozyme fragments [HR(0−6) or HR(6−0)] were proposed to be different from each other. Complex 3 may adopt a more compact conformation than complex 2. If the linker (A6) is inserted at the side of domain II as in the case of complex 2 in Figure 5, the two domains may be distant from each other (Figure 7a). However, if the hexa-adenylate

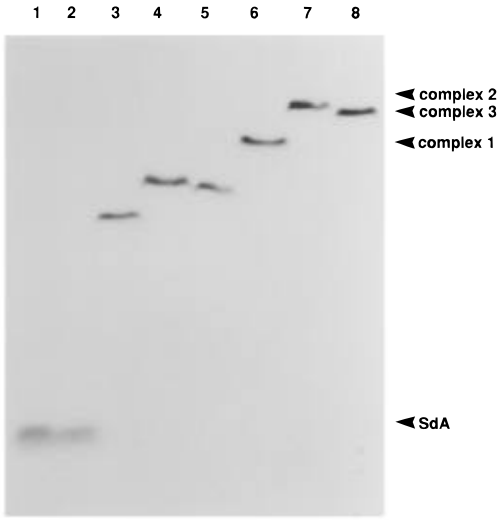



FIGURE 6: Autoradiogram of nondenaturing gel analysis. Complex formation was assayed by combining the 5′-end-labeled, uncleavable substrate analog SdA with ribozyme fragments [R21, RA6, HR-(0−6), HR(6−0), RA6−R21, HR(0−6)−R21, or HR(6−0)−R21] as described previously (Chowria & Burke, 1992; Komatsu et al., 1994). Lane 1, SdA; lane 2, SdA−R21; lane 3, SdA−RA6; lane 4, SdA−HR(0−6); lane 5, SdA−HR(6−0); lane 6, complex 1; lane 7, complex 2; lane 8, complex 3.

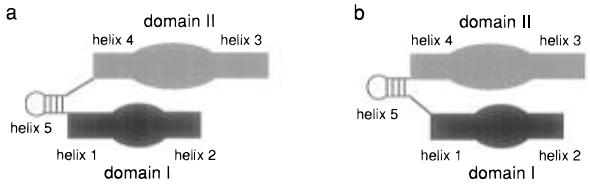



FIGURE 7: Illustration of the predicted conformations of the bent hairpin ribozyme. Conformation of the bent hairpin ribozyme with longer L1 linker (a) and with longer L2 linker (b). Conformation a is inactive by mismatching of the two domains. In conformation b, the two domains are located in favorable positions.

is linked with the smaller domain I (complex 3), the two domains seem to interact easily, as shown in Figure 7b, which shows the similar relative relationship of the two domains in the wild-type hairpin ribozyme, because helix 2 and helix 3 are covalently connected (Figure 1a).

Genomic and antigenomic strands of the hepatitis delta virus can cleave RNAs catalytically, and have been suggested to have pseudoknot-like structures (Perrota & Been, 1991). These ribozymes have four stems and exhibit a complicated folding. A stem (stem IV) and a loop of the hepatitis delta virus ribozymes seem to play a similar role as the helix 5 of the bent ribozyme used here (Figure 5), because both stem−loops seem to stabilize the folding structure of the two domains. It is possible that these multiply folded structures are not favorable for the turnover reaction of the substrate. On the other hand, the wild type and the reversely joined ribozyme may have advantages in binding to substrates, due to the equilibrium between the bent and linear forms. The linear form may be favorable for binding with the substrate, because the binding cannot be inhibited. Then the complex forms a bent conformation by a loop−loop interaction. These conformational changes may enable the wild-type ribozyme to have efficient turnover. The turnover ability of the stemmed ribozymes was found to be low, but the chemical step of cleavage seemed to be high, due to fixed active conformations. These results suggest that the internal loop−loop interaction required for the cleavage was conserved in the present stemmed ribozymes, and these com-

plexes may be suitable for NMR or X-ray structural analysis to resolve the loop−loop interaction.

## CONCLUSION

We have constructed ribozymes with two domains replaced (reversely joined ribozymes) and have proved that these ribozymes were still active (Komatsu et al., 1995). In this report, we modified the reversely joined ribozymes by separating the domains at the linker region, but combined them by linking the complementary arms as an anchor. These ribozymes had cleavage activities at low magnesium ion concentrations, by the association of the two domains in solution. Although the domain without the complementary anchor regions showed weak cleavage activity, the cleavage activity increased by the addition of the anchor region, probably due to an easier association of the two domains (Figure 2). When adenosine linkers were inserted between the anchor region and the catalytic domains, the cleavage activities increased, depending on the number of linker bases. These ribozymes (Figure 2) seemed to always maintain the bent conformation, which may not be advantageous for the binding of a substrate strand. The ribozyme complexes containing a stem−loop (complexes 2 and 3, Figure 5) were tested for their cleavage activities under multiple turnover conditions. Complex 3 was found to be active, in contrast to complex 2, and it was shown by native gel analysis that the conformations of these complexes were different from each other. These results suggest that the relative locations of the two domains should be similar to the wild type.

In conclusion, the present results indicate that the bent conformation is essential during the cleavage reaction, but may be not favorable for the binding and release of substrates and products. From these experiments, a hypothetical mechanism for a higher turnover number of the natural hairpin ribozyme may be derived. The wild-type hairpin ribozyme is able to bind to a substrate in the linear form, and then the two domains interact by transforming to a bent structure for catalytic cleavage. For the release of the cleaved products, the molecule may again change its conformation.

The modified hairpin ribozymes described in this paper are rather rigid to react as an enzyme, but are useful for structural studies of loop−loop interactions in RNA and for the design of new ribozymes.

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BI960627N