Loop-Size Variation To Probe a Bent Structure of a Hairpin Ribozyme

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Abstract: A two-stranded hairpin ribozyme, derived from the catalytic center of the negative strand of the tobacco ring spot virus satellite RNA, catalyzes the cleavage and joining of RNA fragments. In order to probe the bent structure in hairpin ribozymes, complexes were constructed by joining a substrate to the shorter strand of this ribozyme, using multiple units of 1,3-propanediol phosphate at the junction. Active conformations of the complex, in the presence of magnesium ions for substrate cleavage, were estimated by increasing the number of non-nucleotidic linkers. Significant cleavage was observed in the molecule with three linker units and was relatively increased in molecules bearing 4 and 5 linkers. Complexes with 7, 10, and 13 linkers showed cleavage rates of 3.6-, 7.3-, and 8.7-fold of that for the complex with 5 linkers. The effects of the magnesium chloride concentration were greater in molecules with 5 and 7 linkers as compared to those with 10 and 13. Extra space in the junction was shown to be required to form an active structure of the ribozyme–substrate complex. A bent structure in the hairpin ribozymes caused by the extra space has been proposed. A model of the complex with a bend was constructed by joining the junctions with linkers.

Introduction

The three-dimensional structures of RNA are thought to be responsible for their functions. However, chemical approaches to study RNA structure are rather limited. Bent structures of RNA have attracted much attention for their role in molecular recognition between nucleic acids and proteins.[1] The catalytic center of a self-cleaving domain in the minus strand of tobacco ring spot virus satellite RNA (-sTobRV) forms a hairpin-like secondary structure.[2-4] The domain comprises an enzyme (E50) and a substrate (UGACAGUCCUGUUUC, S1) including loop 6, as shown in Figure 1a. Cleavage occurs by transesterification of the 3′-phosphate of adenosine (an arrow in Figure 1) in the presence of magnesium ions. The tertiary structure of this active domain has yet to be solved. We have previously shown that E50 can be divided into two fragments (E29, 21, and E30, 21) at loop 3 without a loss in the cleavage activity.[5,6] A bent structure is assumed to exist in E50 at the junction (loop 5), since a large RNA duplex is inserted in the natural RNA.[4,7] Mutagenesis experiments have suggested that loop 2 is located near the cleavage site, and a mutant hairpin ribozyme containing ACCCCC at loop 5 has been shown to be active.[8] The conformations of the oligonucleotide loops are restricted by base sequences, due to the interactions of the nucleobases.[9-11] However, non-nucleotidic tethers have been introduced in oligodeoxyribonucleotides and have been found to increase the flexibility of DNA fragments.[12-15] We now report a new ribozyme analog that proves that a bent structure is the essential factor for its activity. A series of ribozymes was constructed by the insertion of units of a 1,3-propanediol phosphate linker[12] E(PPL)$_n$S (n = 0∼13), and the E(PPL)$_n$S is then combined with E30 (Figure 1b). E(PPL)$_n$S molecules containing more than three units of the linker formed active complexes and were cleaved, as shown by the arrow. The cleavage, which required magnesium ions, was increased as the number of linkers increased, and the effect of magnesium ions was greatest in molecules containing five and seven linker units. The cleavage of a control natural molecule, consisting of E19–S1–E30 (Figure 1c), was tested for comparison. The rates for E(PPL)$_4$S–E30 and E(PPL)$_5$S–E30 cleavage at different Mg^{2+} concentrations suggested that the flexibility of the molecule affects the formation of active conformations. A molecular model of the hairpin ribozyme with a possible bend in the molecule was constructed.

Results and Discussion

Cleavage of E(PPL)$_n$S–E30 (n = 0∼5). A series of E(PPL)$_n$S oligonucleotides (Figure 1b, n = 0–5) and E30 were synthesized by the standard phosphoramidite method. Linkers were inserted using 1-*O*-(4,4′-dimethoxytrityl)-3-*O*-[(*N*,*N*-diisopropylamino)-(β-cyanoethoxy)phosphino]-1,3-propanediol as a coupling unit. The oligonucleotides were purified by reverse-phase and anion-exchange chromatographies. The 3′-end of E(PPL)$_n$S was labeled by the addition of [^{32}P]pCp, and the denatured product was mixed with E30. Cleavage reactions were performed in the presence of 12 mM magnesium chlorixde at 37 °C. Although the optimal reaction temperature of the two-stranded ribozyme was 32 °C,[5] E(PPL)$_n$S–E30 molecules were found to be more active at 37 °C than at 32 °C (data not shown). A tenfold excess of E30 (810 nM) was used to prevent multiple binding of E(PPL)$_n$S to E30.

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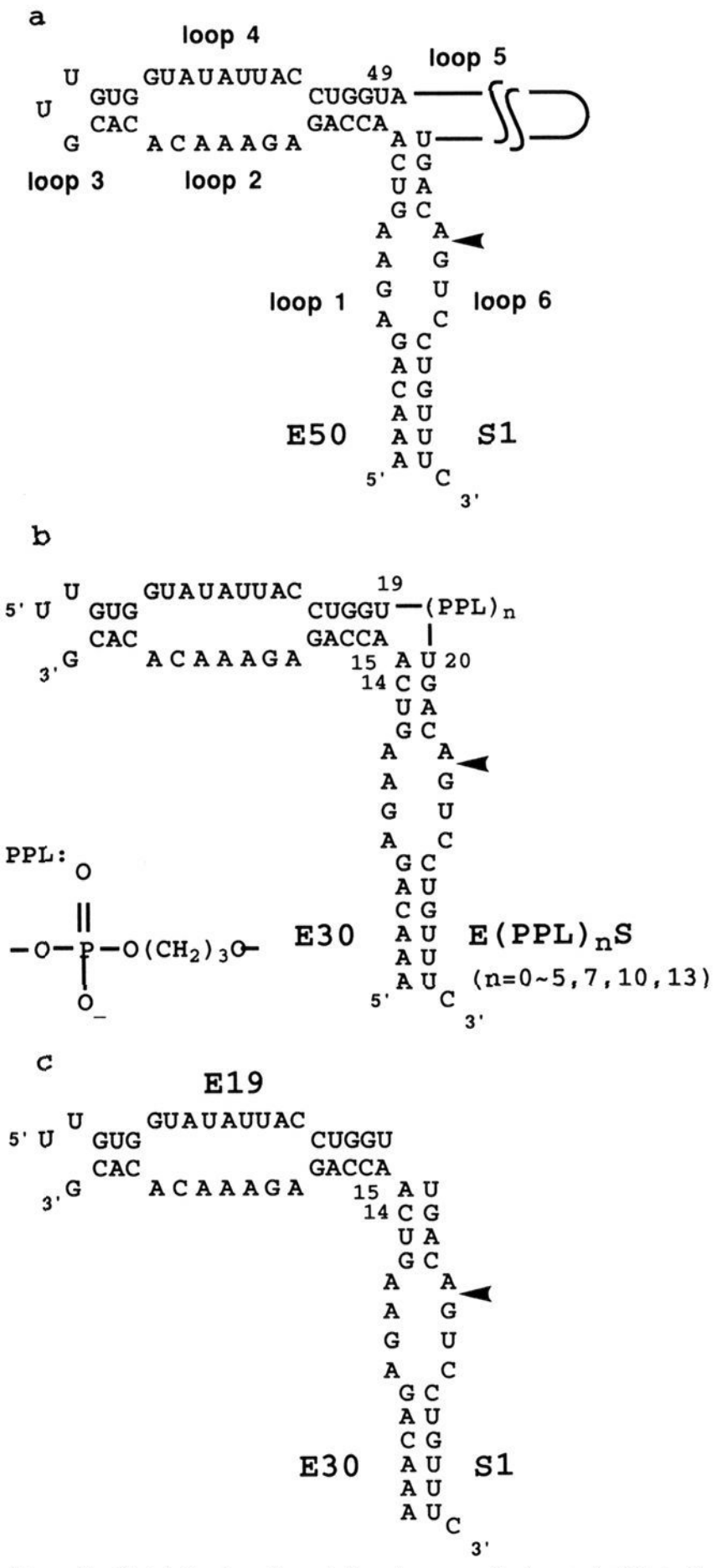

Figure 1. Catalytic domain and the cleavage site (arrow) of hairpin ribozymes. (a) Postulated secondary structure of the catalytic domain of a hairpin ribozyme derived from sTobRV(–), which contains six internal loops.[4] Loop 5 consists of 121 bases. (b) Analog of the hairpin ribozyme (E(PPL)$_n$S–E30) containing different numbers of PPL units instead of loop 5. (c) Control natural complex, E19–S1–E30, which is separated at the junction of E19–S1 and at loop 3.

The same cleavage rates were obtained with a 1.5-fold equivalent of E30 (122 nM). The products from E(PPL)$_n$S cleavage at different time intervals (0–40 min) were analyzed by polyacrylamide gel electrophoresis. The results are shown in Figure 2. Complexes with shorter linkers, E(PPL)$_n$S (n = 0–2), did not show any significant cleavage after 40 min under these conditions. The cleavage of E(PPL)$_3$S yielded 7.3% product, and E(PPL)$_5$S–E30 was found to be more than 60% cleaved after 40 min.

Complex formation of E(PPL)$_n$S (n = 0–5) with E30 and of S1 with E50 was confirmed by gel retardation.[16,17] Labeled

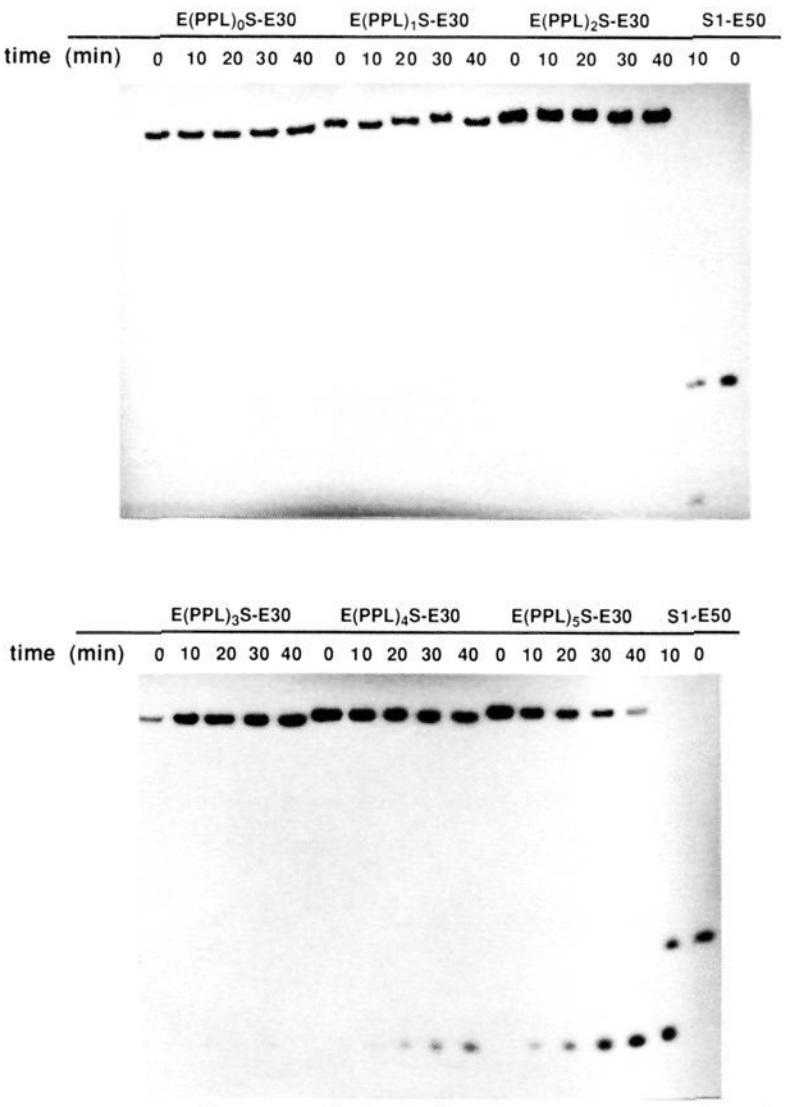

Figure 2. Autoradiogram of the 15% polyacrylamide gel analysis (containing 8 M urea) of the cleavage reaction of the E(PPL)$_n$S–E30 complex (n = 0–5). This reaction was carried out under the conditions indicated in the methods. To obtain a cleaved marker, 3′-labeled S1 (15 mer ^{32}pCp, 81 nM) was treated with E50 (32 nM).

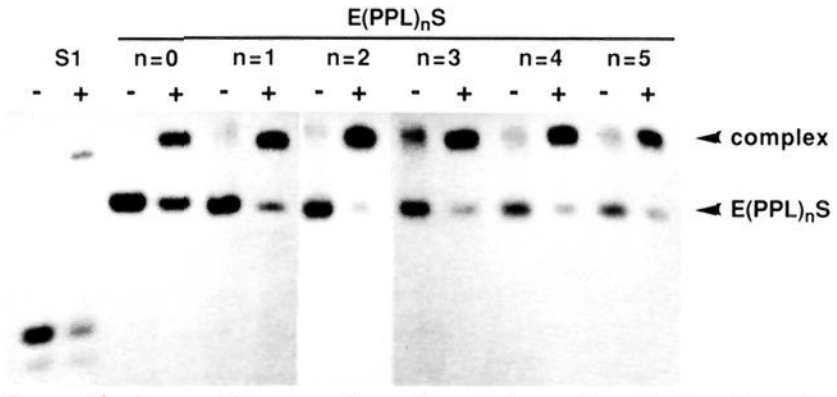

Figure 3. Autoradiogram of nondenaturing gel analysis. Complex formation was assayed by combining ^{32}P-end-labeled E(PPL)$_n$S (n = 0–5) or S1 with nonlabeled ribozyme: +, ribozyme is present; –, no ribozyme is present.

E(PPL)$_n$S (81 nM), in the presence of a 1.5-fold excess of E30 (122 nM), was subjected to electrophoresis under nondenaturing conditions at 4 °C (Figure 3). At least half of the E(PPL)$_0$S behaved as a complex with E30. The remaining combinations showed retarded migration, although a small amount of E(PPL)$_n$S was detected as a single strand in each case. The number of Watson–Crick-type hydrogen bonds in all complexes is the same, even in the less stable E(PPL)$_0$S–E30. The difference is the cleavage activity of E(PPL)$_n$S–E30 did not result from a difference in the stability of the complex. The complex of S1 with E50 traveled faster than E(PPL)$_0$S–E30, probably because S1–E50 had the intact loop 3, while E(PPL)$_0$S–E30 had dangling ends in what was previously loop 3. In the absence of E30, E(PPL)$_0$S did not show any retarded compounds, in contrast to the case of E(PPL)$_n$S (n = 1–5), which formed aggregates that traveled slightly slower than the hetero complex.

The effect of magnesium ions was tested by comparing the rate constants for E(PPL)$_n$S–E30 (n = 0–5) at 12 mM and 100

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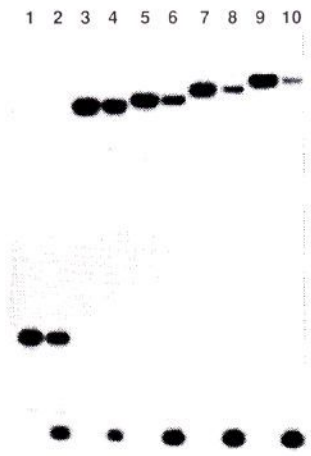

Figure 4. Autoradiogram of 15% polyacrylamide gel analysis of the cleaved products of E(PPL)$_n$S–E30 (n = 5, 7, 10, and 13) and E19–S1–E30 after a 10-min reaction in the buffer containing 12 mM $MgCl_2$. The final concentrations of E19, S1, and E30 were 81, 81, and 810 nM, respectively: lane 1, E19–S1; lane 2, E19–S1–E30; lane 3, E(PPL)$_5$S; lane 4, E(PPL)$_5$S–E30; lane 5, E(PPL)$_7$S; lane 6, E(PPL)$_7$S–E30; lane 7, E(PPL)$_{10}$S; lane 8, E(PPL)$_{10}$S–E30; lane 9, E(PPL)$_{13}$S; lane 10, E(PPL)$_{13}$S–E30.

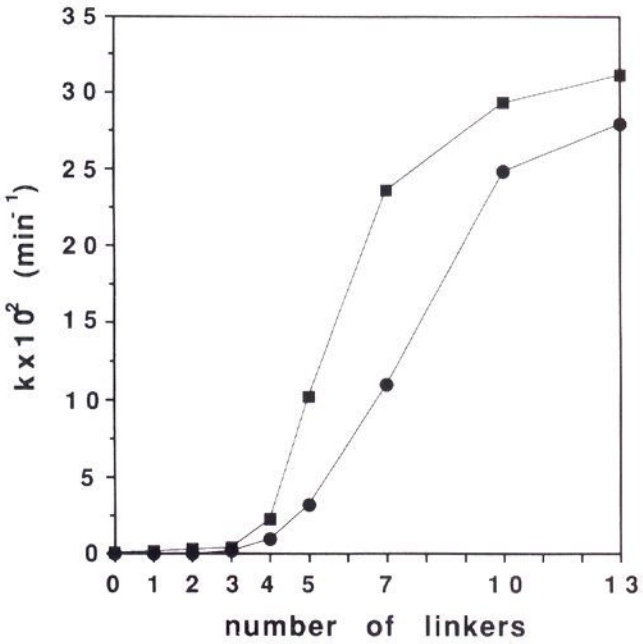

Figure 5. Plots of rate constant versus number of linkers in the presence of 12 mM $MgCl_2$ (solid circles) and 100 mM $MgCl_2$ (solid squares).

mM Mg^{2+} concentrations. The cleavage rates for E(PPL)$_4$ and E(PPL)$_5$S with increasing concentrations of magnesium ions (4–120 mM) were measured. The reaction was accelerated at the higher Mg^{2+} concentration. E(PPL)$_5$S–E30 showed a higher value than E(PPL)$_4$S–E30 at all magnesium ion concentrations. The E(PPL)$_4$S complex never seemed to achieve a more active conformation as compared to the E(PPL)$_5$S complex. These complexes showed maximum rates of ca. 70 mM $MgCl_2$. Even at 120 mM Mg^{2+}, the cleavage rate of the E(PPL)$_4$S complex (2.4×10^{-2} min^{-1}) did not reach the value of the E(PPL)$_5$S complex at 20 mM Mg^{2+} (2.8×10^{-2} min^{-1}). These results indicate that cleavage is not dependent on the affinity of the complex for the cation. The conformation of the complex and the flexibility of the molecule, which result from the insertion of a longer linker, are the important factors for the cleavage of the ribozyme. The association constants (K_a) were calculated from a Hill plot.[18,19] A value of K_a = 37 M^{-1} was obtained for E(PPL)$_4$S–E30, and for E(PPL)$_5$S–E30, a value of K_a = 27 M^{-1} was obtained.

Cleavage of E(PPL)$_n$S–E30 (n = 7, 10, and 13). In order to investigate further the effect of linker lengths in these molecules, complexes containing 7, 10, or 13 linkers were constructed and tested for their activity together with that of a control natural molecule, E19–S1–E30, shown in Figure 1c. The results a cleavage reaction at 10 min in 12 mM $MgCl_2$ are shown in Figure 4. The cleavage activity increased dramatically up to a linker number of 10. The difference in cleavage rates between E(PPL)$_{10}$S and E(PPL)$_{13}$S was small. Figure 5 shows the rate constants for the ribozyme analogs with different numbers of linker units, tested at either 12 or 100 mM $MgCl_2$. The rate

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Table 1. Relative Rates of Cleavage[a]

number of linkers	k_{rel} (12 mM $MgCl_2$)	k_{rel}[c] (100 mM $MgCl_2$)
0	N.D.[b]	0.03
1	N.D.	0.05
2	N.D.	0.10
3	0.06	0.12
4	0.30	0.71
5	1.0	3.2
7	3.6	7.4
10	7.3	9.2
13	8.7	9.8
E19–S1	1.7	8.3

[a] The reactions were carried out using concentrations of 81 nM 3′-end-labeled substrates (E(PPL)$_n$S–^{32}pCp and S1–^{32}pCp–E19) in the presence of 810 nM E30 at 37 °C. The reaction buffer contained 40 mM Tris-HCl (pH 7.5), 2 mM spermidine–3HCl, and either 12 or 100 mM $MgCl_2$. The cleavage rates were determined from the percentage of the substrates remaining at different time intervals. [b] N.D.: not detected. [c] k_{rel}: relative cleavage rate with respect to that of E(PPL)$_5$S–E30.

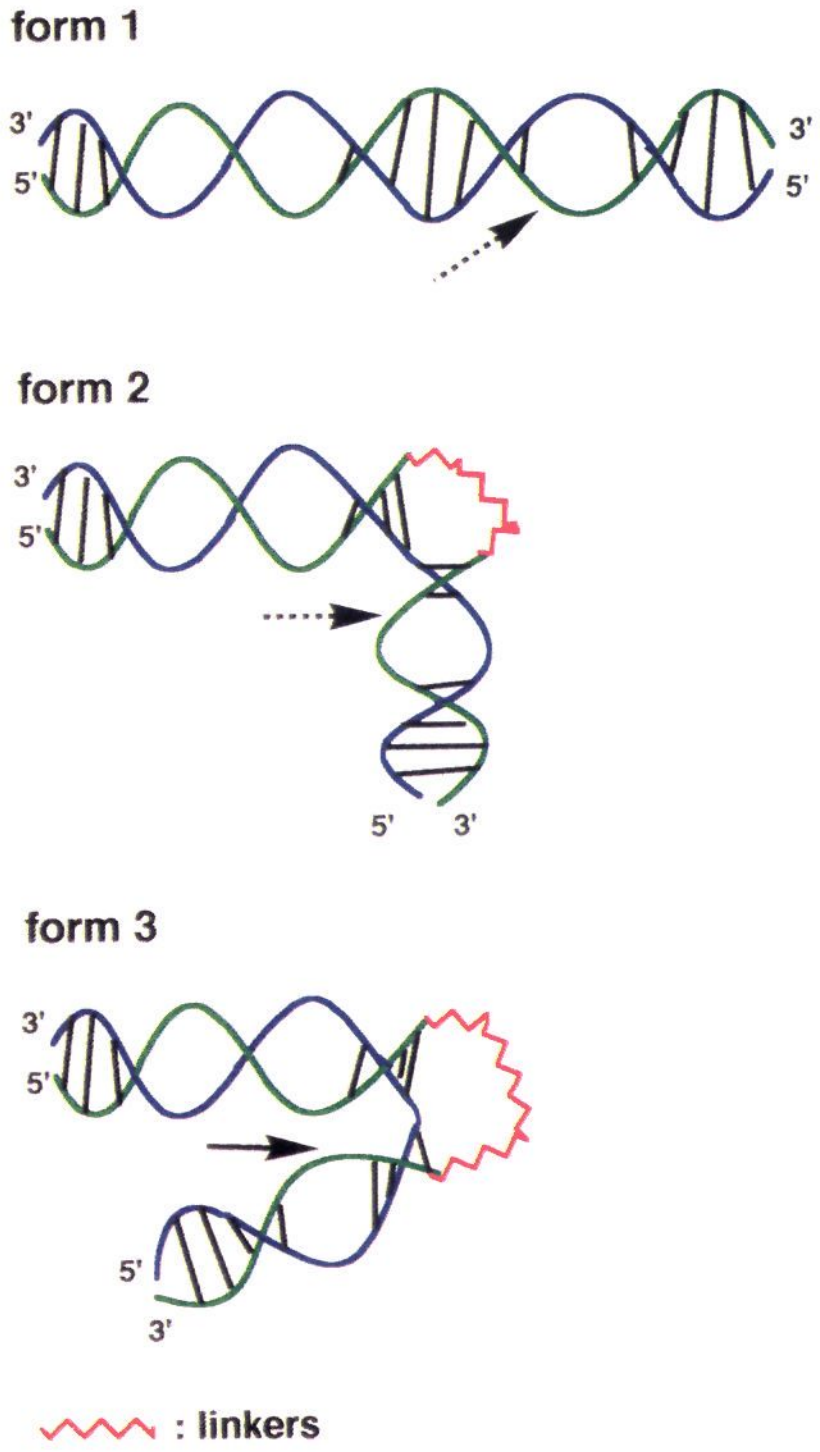

Figure 6. Schematic drawings of the possible conformations of the hairpin ribozyme with different linker lengths: form 1, E(PPL)$_n$S–E30, no linker complex; form 2, complexes with the shorter linkers; form 3, the active conformation with sufficient linker lengths for cleavage. Dotted arrows indicate the cleavage site in the less active forms. The solid arrow indicates the cleavage site: E(PPL)$_n$S, green and red (PPL); E30, blue.

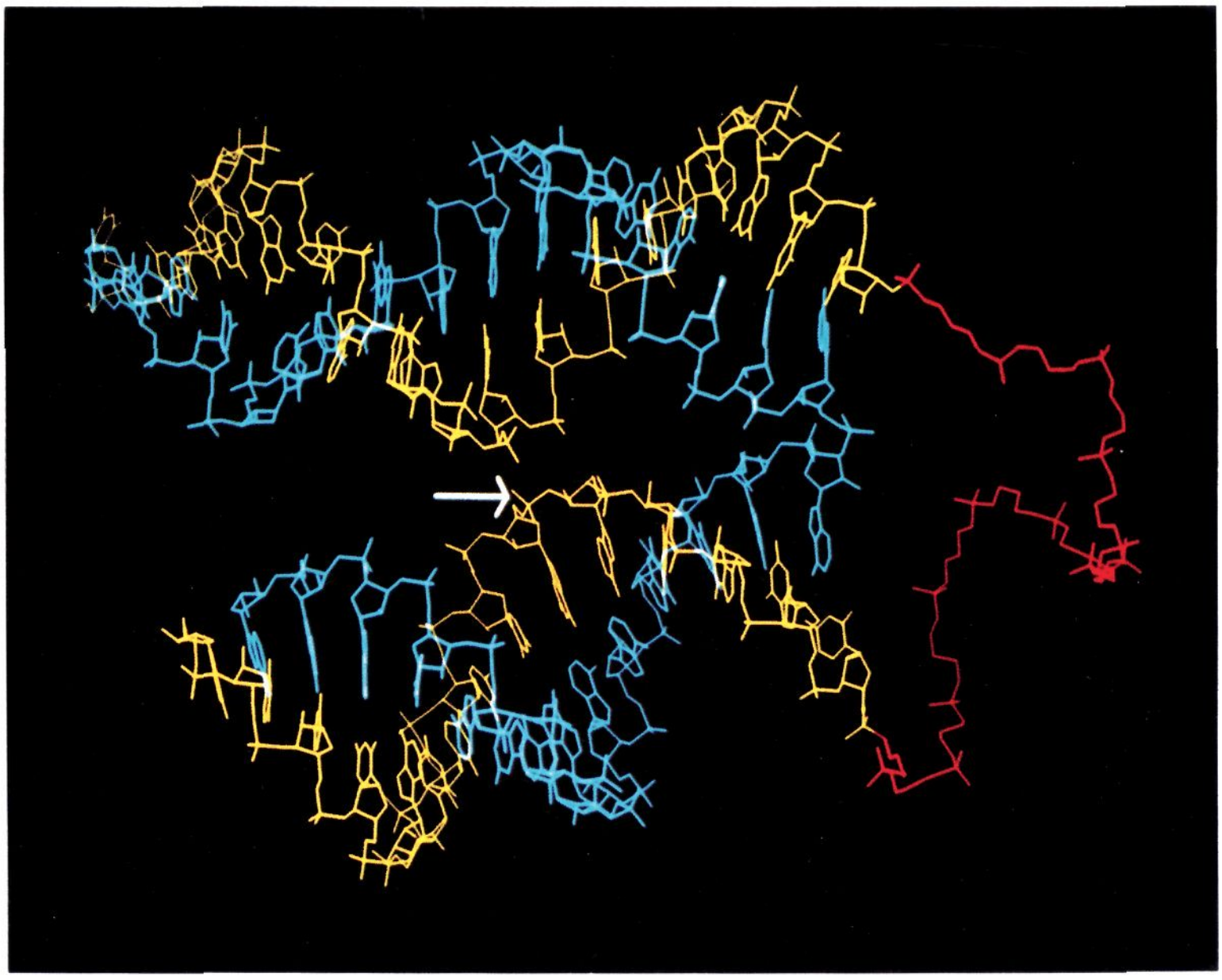

Figure 7. Molecular model of the mutant hairpin ribozyme E(PPL)$_{13}$S–E30 (connected at the 3′-end of E30 and the 5′-end of E(PPL)$_{13}$S) from two perspectives: E(PPL)$_{13}$S, yellow and red (PPL); E30, blue. The white arrow indicates the cleavage site.

increased with the number of linkers and showed larger values at 100 mM $MgCl_2$. However, the effect of the $MgCl_2$ concentration was smaller in molecules with 10 and 13 linkers as compared to those with 5 and 7 linkers. Table 1 lists the relative rate constants of the cleavage at 12 and 100 mM $MgCl_2$ with respect to that of E(PPL)$_5$S at 12 mM $MgCl_2$. The control molecule, E19–S1–E30, showed a lower cleavage activity ($k = 5.3 \times 10^{-2}$ min^{-1}) than that of E(PPL)$_7$S–E30 at 12 mM $MgCl_2$. However at 100 mM $MgCl_2$, the nonlinked control molecule exhibited a faster rate ($k = 27 \times 10^{-2}$ min^{-1}) than that of E(PPL)$_7$S–E30.

Several bases in loops 2 and 4 are required for the cleavage activity.[8,20] These loops are thought to interact with loops 1 and 6, and the active domain may be formed by these four loops. The correlation of the number of linkers with the cleavage activity suggests that a bent structure, formed by the insertion of longer linkers at the junction, may be required for achieving an active conformation for cleavage.

Figure 6 shows a series of schematic drawings of the possible bend in these hairpin ribozymes. E(PPL)$_0$S–E30, the molecule with no linker, forms a continuous helix, and the four internal loops are not able to interact with each other (Figure 6, form 1). Consequently, E(PPL)$_0$S–E30 may not form an active conformation and does not exhibit cleavage activity. The less active molecules with 1–3 linkers, E(PPL)$_n$S–E30 (n = 1, 2, and 3), may have a structure in which loop 2 or loop 4 cannot efficiently access loop 1 or loop 6 (Figure 6, form 2), so very few molecules could form an active conformation (Figure 6, form 3). When 5 or 7 linkers are inserted into E(PPL)$_n$S, some molecules might exist in the active form. Although the four loops in these complexes may be able to interact, the intraction may still be constrained by the linkers. These loops in the molecules with 10 or 13 linkers, E(PPL)$_n$S–E30 (n = 10 or 13), may be able to access each other easily, and this flexibility led to higher activity, even at low magnesium ion concentrations. The flexibility at the junction may increase the proportion of molecules with the active conformation (form 3). Although the control molecule, E19–S1, has greater flexibility, it had lower activity as compared to complexes with 10 or 13 linkers. These results, together with the effect of the magnesium ion concentration, suggest that E19–S1–E30 is too flexible to have maximal activity at low magnesium ion concentrations. However, at high magnesium ion concentrations, it tends to be stabilized to form an active species. Molecules with 10 or 13 linkers require a lower magnesium ion concentration for their cleavage. The longer linkers might facilitate the bent structure of the ribozyme.

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Model Building

A three-dimensional model of the S1–E50 complex, which allows interactions of the internal loops, was built. The distance between the 5′-end of S1 and the 3′-end (U49) of E50 was estimated to be ca. 30 Å, and these ends were able to be connected by more than five linkers. However, the proportion of molecules with the active conformation is supposed to decrease with five units of the linker (E(PPL)$_5$S–E30), due to the constraint. E(PPL)$_n$S–E30 with longer linkers will be able to form an active species that has a possible bend. Figure 7 shows a model of E(PPL)$_{13}$S–E30 with the proposed bend.

The entire backbone structure, except for GUU loop 3, was first built on the basis of the A-form RNA structure.[21] The GUU loop 3 backbone structure was taken from the TΨC loop of the yeast tRNA^{ASP}.[22] Bends in the junctions of A14 and A15,

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as well as between U19 and U20 (Figure 1b) of the $E(PPL)_{13}S$–E30 molecule with the PPL, were introduced by manipulation with the molecular graphics program HYDRA (Polygen, Waltham, MA). Using the molecular mechanics program, PRESTO,[23] with an AMBER all-atom force field,[24] the structure of the mutant hairpin ribozyme was optimized and bad contacts were minimized by the conjugate gradient method.

Conclusion

A series of hairpin ribozymes that contain increasing numbers of 1,3-propanediol phosphates at the site of a postulated bent position were synthesized. The cleavage in the presence of magnesium ions showed that the rate increased with the number of linker units and that the activity of the molecule with 4 linkers could not be increased to the level of that with 5 linkers, even at high concentrations of magnesium chloride. The effect of magnesium ions on the cleavage is greater in reactions of molecules with 5 and 7 linkers than that in those with 10 and 13 linkers. These results suggest that the cleavage activity of the ribozyme depends on its flexibility, which permits a favorable conformation for the access of either loop 2 or loop 4 to the cleavage site. Magnesium ions assist in the formation of the active structure, and a longer non-nucleotidic linker (10 or 13) may provide flexibility that results in a favorable conformation for cleavage. These contacts can be explained by a bent structure. By constructing a model of a ribozyme (Figure 7), the distance between U19 and U20 was estimated to be ca. 30 Å, which was found to be linked by more than 5 units of the propanediol phosphate linker. The use of abasic linkers, instead of a nucleotide loop, in RNA molecules provides a method to probe a bend without the influence of interactions from nucleic acid base moieties.

Experimental Section

Preparation and Labeling of Oligoribonucleotides. 1-*O*-(4,4′-dimethoxytrityl)-3-*O*-[(*N*,*N*-diisopropylamino)(β-cyanoethoxy)phosphino]-1,3,-propanediol was prepared as described.[12] Oligoribonucleotides were prepared by the phosphoramidite method using 2′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-(dimethoxytrityl)-3′-*O*-[(*N*,*N*-diisopropylamino)(β-cyanoethoxy)phosphino]nucleosides, which were purchased from Milligen and deblocked as described.[6,25] A DNA synthesizer, Applied Biosystems Model 381A, was employed for coupling reactions. Oligonucleotides were purified by reverse-phase and anion-exchange HPLC using columns from Inertsil ODS-2, 10 mm i.d. × 300 mm or 4.6 mm i.d. × 250 mm (GL Sciences), and TSK gel DEAE-2SW, 4.6 mm i.d. × 250 mm (Tosoh). The 3′ termini of $E(PPL)_nS$ and S1 were labeled with [^{32}P]pCp and RNA ligase using conditions described previously.[6]

Cleavage Reaction. $E(PPL)_nS$ (0.162 μM, 10 μL) and E30 (1.62 μM, 40 μL) in cleavage buffer (40 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 2 mM spermidine–3HCl) were separately heated at 65 °C for 2 min and then immediately transferred to an ice bath. To start the reaction, the E30 solution (10 μL) was added to the solution of $E(PPL)_nS$ (10 μL) and kept at 37 °C unless otherwise specified. The reaction was stopped by the addition of 50 mM Na_2EDTA, and the products were analyzed by electrophoresis. Cleavage rates were estimated by measuring radioactivities using a Bioimaging analyzer (FUJIX BAS 2000).

Nondenaturing Gel Electrophoresis. Nondenaturing gel electrophoresis was carried out using 15% polyacrylamide (acrylamide/bisacrylamide, 19:1) gels (30 × 25 × 0.05 cm) in 40 mM Tris–acetate (pH 7.5) and 12 mM magnesium acetate buffer at 4 °C. Unlabeled E30 or E50 (81 nM) and 3′ terminus labeled $E(PPL)_nS$ or S1 were combined in 40 mM Tris-HCl (pH 7.5), heated to 95 °C for 1 min, and allowed to cool to room temperature. An equal volume of 30% glycerol was added, and samples were loaded onto a 15% nondenaturing polyacrylamide gel.

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